Journal of NEUROCHEMISTRY

VOLUME 5, 1959/60

OL.
5
9/60

Editorial Board

R. V. COXON (Oxford)
V. ENGELHARDT (Moscow)
A. ENGSTRÖM (Stockholm)
J. FOLCH-PI (Waverley, Mass.)
S. S. KETY (Bethesda, Md.)
E. KLENK (Cologne)
C. LONG (London)
A. POPE (Waverley, Mass.)
D. RICHTER (Cardiff)
R. ROSSITER (London, Ont.)
W. M. SPERRY (New York)
M. VOGT (Cambridge)
H. WAELSCH (New York)

Advisory Board

G. B. ANSELL (Birmingham)
W. BARGMANN (Kiel)
J. BENOIT (Paris)
D. BOVET (Rome)
F. BRINK, JR. (New York)
J. N. CUMINGS (London)
R. M. C. DAWSON (Cambridge)
J. ELKES (Washington)
K. A. C. ELLIOTT (Montreal)
O. ERÄNKÖ (Helsinki)
V. ERSPAMER (Parma)
U. S. von EULER (Stockholm)
W. FELDBERG (London)
H. FERNÁNDEZ-MORÁN (Boston)
L. B. FLEXNER (Philadelphia, Pa.)
J. H. GADDUM (Cambridge)
R. W. GERARD (Ann Arbor, Mich.)
G. W. HARRIS (London)
C. HEBB (Cambridge)
H. HYDÉN (Göteborg)
Sir HANS KREBS (Oxford)
O. LINDBERG (Stockholm)
R. LORENTE de NÓ (New York)
O. H. LOWRY (St. Louis, Mo.)
H. McILWAIN (London)
A. v. MURALT (Berne)
A. V. PALLADIN (Kiev)
K. R. PORTER (New York)
J. H. QUASTEL (Montreal)
E. ROBERTS (Duarte, Calif.)
B. SCHARRER (New York)
F. O. SCHMITT (Cambridge, Mass.)
S. G. WAELSCH (New York)
P. WEISS (New York)

Pergamon Press

Oxford • New York *Paris • Los Angeles*

JOURNAL OF NEUROCHEMISTRY

Editors:

R. V. COXON: *University Laboratory of Physiology, Oxford.*
V. ENGELHARDT: *The Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.*
A. ENGSTRÖM: *Department of Medical Physics, Karolinska Institutet, Stockholm, Sweden.*
J. FOLCH-PI: *McLean Hospital, Waverley, Mass., U.S.A.*
S. S. KETY: *Department of Health, Education and Welfare, National Institutes of Health, Bethesda* 14, *Md., U.S.A.*
E. KLENK: *Physiologisch-Chemisches Institut der Universität, Köln, Germany.*
C. LONG: *Royal College of Surgeons, Lincoln's Inn Fields, London W.C.*2.
A. POPE: *McLean Hospital, Waverley, Mass., U.S.A.*
D. RICHTER: *Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff, England.*
R. ROSSITER: *Department of Biochemistry, University of Western Ontario, London, Canada.*
W. M. SPERRY: *New York State Psychiatric Institute, New York* 32, *N.Y., U.S.A.*
M. VOGT: *Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge, England.*
H. WAELSCH: *New York State Psychiatric Institute, New York* 32, *N.Y., U.S.A.*

VOL
5
1959/

Publishing Offices: Headington Hill Hall, Oxford (*Oxford* 64881)

Issued in parts. Subscription per volume (*A*) £6 (*U.S.A.* $17.00) *including postage;* (*B*) *for individual subscribers who write direct to the publisher certifying that the journal is for their private use,* £3 10*s.* (*U.S.A.* $10.00) *including postage.*

Copyright © 1959/60, *Pergamon Press Ltd.*

LIST OF CONTENTS

NUMBER 1

R. BALÁZS and J. R. LAGNADO: Glycolytic activity associated with rat brain mitochondria . 1

RICHARD E. MCCAMAN and ELI ROBINS: Quantative biochemical studies of Wallerian degeneration in the peripheral and central nervous systems—I 18

RICHARD E. MCCAMAN and ELI ROBINS: Quantative biochemical studies of Wallerian degeneration in the peripheral and central nervous systems—II 32

J. -E. EDSTRÖM: Ribonucleic acid changes in the motoneurons of the frog during axon regeneration 43

A. GIUDITTA and H. J. STRECKER: Alternate pathways of pyridine nucleotide oxidation in cerebral tissue 50

MICHAEL B. SPORN, WESLEY DINGMAN, ALFRED DEFALCO and ROBERT K. DAVIES: The synthesis of urea in the living rat brain 62

JULIUS AXELROD, WAYNE ALBERS and CARMINE D. CLEMENTE: Distribution of catechol-O-methyl transferase in the nervous system and other tissues 68

EDWARD GELLER, SAMUEL EIDUSON and ARTHUR YUWILER: Oxidation of *p*-phenylenediamine and adrenaline in enzymic and copper-catalysed reactions 73

EDMUND L. PETERS and DONALD B. TOWER: Glutamic acid and glutamine metabolism in cerebral cortex after seizures induced by methionine sulphoximine 80

HUNTINGTON PORTER and SALLY AINSWORTH: The isolation of the copper-containing protein cerebrocuprein I from normal human brain 91

N. CANAL and A. MAFFEI-FACCIOLI: Reversal of the reserpine-induced depletion of brain serotonin by a monoamine oxidase inhibitor 99

Announcements 101

Papers to be published in future issues 103

NUMBER 2

M. WENDER and MARIAN HIEROWSKI: The concentration of electrolytes in the developing nervous system with special reference to the period of myelination 105

J. KRAWCZYŃSKI, S. WISZNIOWSKA and I. DREWNOWSKA: Incorporation of radioactivity into proteins of the cerebrum, cerebellum and other organs, after the injection of ^{35}S in the form of sodium sulphate 109

M. WOLMAN and SARAH HESTRIN-LERNER: A histochemical contribution to the study of the molecular morphology of myelin sheath 114

M. J. E. ERNSTING, W. F. KAFOE, W. TH. NAUTA, H. K. OOSTERHUIS and C. DE WAART: Biochemical studies on psychotropic drugs—I. The effect of psychotropic drugs on γ-amino-butyric acid and glutamic acid in brain tissue 121

J. MAGNES and SARAH HESTRIN-LERNER: Effect of 5-hydroxytryptophan and serotonin on glucose content and functional activity of the perfused cat brain 128

B. MCARDLE, R. H. S. THOMPSON and G. R. WEBSTER: The action of lysolecithin and of snake venom on whole-cell preparations of brain muscle and liver 135

D. M. ROBERTSON: The paper electrophoretic distribution of soluble proteins in different regions of human brain 145

M. H. APRISON, ARLENE LUKENBILL and WILLIAM E. SEGAR: Sodium, potassium, chloride and water content of six discrete parts of the mammalian brain 150

R. L. FRIEDE: Histochemical investigations on succinic dehydrogenase in the central nervous system—IV. A histochemical mapping of the cerebral cortex of the guinea pig 156

S. ROSENBLATT, J. D. CHANLEY, H. SABOTKA and M. R. KAUFMAN: Interrelationships between electroshock, the blood-brain barrier, and catecholamines 172

A. J. HUDSON, J. H. QUASTEL and P. G. SCHOLEFIELD: The effect of heated snake venom on the phosphate metabolism of the rat spinal cord 177

C. S. SPYROPOULOS: Cytoplasmic pH of nerve fibres 185

A. BONICHON: Developpement de la cholinesterase au niveau du mesencephale chez l'embryon de poulet 195

G. GÁRDOS: Potassium accumulation in guinea pig brain cortex slices 198

R. KATO: Serotonin content of rat brain in relation to sex and age 201

C. F. BAXTER, E. ROBERTS and E. EIDELBERG: γ-Aminobutyric acid and seizure susceptibility in areas of normal cat brain cortex 202

Papers to be published in future issues 206

NUMBER 3

HELEN H. HESS and ALFRED POPE: Intralaminar distribution of cytochrome oxidase activity in human frontal isocortex 207

ALFRED F. HARRIS and ABRAHAM SAIFER: The metachromasy of strandin 218

NORMAN M. SULKIN: The distribution of mucopolysaccharides in the cytoplasm of vertebrate nerve cells 231

C. F. EMANUEL and I. L. CHAIKOFF: Deoxyribonucleic acids of central nervous system, kidney, and spleen: a comparison of some chemical and physical properties 236

WILLIAM G. VAN DER KLOOT: Factor S—a substance which excites crustacean muscle . . 245

LEONARD H. COHEN and WERNER K. NOELL: Glucose catabolism of rabbit retina before and after development of visual function 253

G. PORCELLATI and B. CURTI: Proteinase activity of peripheral nerves during Wallerian degeneration 277

M. KUROKAWA: Metabolic consequences of localized application of electrical pulses to sections of cerebral white matter 283

C. W. M. ADAMS and A. N. DAVISON: The form in which cholesterol occurs in the adult CNS . 293

HOWARD SACHS: Vasopressin biosynthesis—I. *In vivo* studies 297

K. YAGI, T. OZAWA, M. ANDO and T. NAGATSU: The effect of flavin adenine dinucleotide on the electroencephalogram modified by chlorpromazine 304

NUMBER 4

Bo HALLGREN and PATRICK SOURANDER: The non-haemin iron in the cerebral cortex in Alzheimer's disease 307

A. GEIGER, N. HORVATH and Y. KAWAKITA: The incorporation of ^{14}C derived from glucose into the proteins of the brain cortex, at rest and during activity 311

A. GEIGER, Y. KAWAKITA and S. S. BARKULIS: Major pathways of glucose utilization in the brain in brain perfusion experiments *in vivo* and *in situ* 323

S. S. BARKULIS, A. GEIGER, Y. KAWAKITA and V. AGUILAR: A study of the incorporation of ^{14}C derived from glucose into the free amino acids of the brain cortex 339

T. SAMORAJSKI: The application of diphosphopyridine nucleotide diaphorase methods in a study of dorsal ganglia and spinal cord. 349

ROBERT SIMINOFF and PAUL R. SAUNDERS: Effect of morphine on the uptake and oxidation of glucose by rabbit brain 354

H. NARUSE, M. KATO, M. KUROKAWA, R. HABA and T. YABE: Metabolic defects in a convulsive strain of mouse 359

PAUL HOCHSTEIN and GERALD COHEN: The inhibitory effects of quinones and dihydric phenols on glucose metabolism in sub-cellular systems of brain 370

R. U. MARGOLIS, S. S. BARKULIS and A. GEIGER: A comparison between the incorporation of ^{14}C from glucose into N-acetyl-L-aspartic acid and aspartic acid in brain perfusion experiments 379

ALFRED F. HARRIS and ABRAHAM SAIFER: The interaction of strandin with basic proteins . . 383

Announcement 385

Papers to be published in future issues 386

VOL.
5
1959/6

AUTHOR INDEX TO VOLUME 5

ADAMS C. W. M. 293
AGUILAR V. 339
AINSWORTH S. 91
ALBERS W. 68
ANDO M. 304
APRISON M. H. 150
AXELROD J. 68
BALÁZS R. 1
BARKULIS S. S. 323, 339, 379
BAXTER C. F. 203
BONICHON A. 195
CANAL N. 99
CHAIKOFF I. L. 236
CHANLEY J. D. 172
CLEMENTE C. D. 68
COHEN G. 370
COHEN L. H. 253
CURTI B. 277
DAVIES R. K. 62
DAVISON A. N. 293
DEFALCO A. 62
DINGMAN W. 62
DREWNOWSKA I. 109
EDSTRÖM J. -E. 43
EIDELBERG E. 203
EIDUSON S. 73
EMANUEL C. F. 236
ERNSTING M. J. E. 121
FRIEDE R. L. 156
GÁRDOS G. 198
GELLER E. 73
GEIGER A. 311, 323, 339, 379
GIUDITTA A. 50
HABA R. 359
HALLGREN B. 307
HARRIS A. F. 218, 383
HESS H. H. 207
HESTRIN-LERNER S. 114, 128
HIEROWSKI M. 105
HOCHSTEIN P. 370
HORVATH N. 311
HUDSON A. J. 177
KAFOE W. F. 121
KATO M. 359
KATO R. 202
KAUFMAN 172
KAWAKITA Y. 311, 323, 339
KRAWCZYŃSKI, J. 109
KUROKAWA M. 283, 359
LAGNADO J. R. 1
LUKENBILL A. 150
MAFFEI-FACCIOLO A. 99
MAGNES J. 128
MARGOLIS R. U. 379
MCARDLE B. 135
MCCAMAN R. E. 18, 32
NAGATSU T. 304
NARUSE H. 359
NAUTA W. Th. 121
NOELL W. K. 253
OOSTERHUIS H. K. 121
OZAWA T. 304
PETERS E. L. 80
POPE A. 207
PORCELLATI G. 277
PORTER H. 91
QUASTEL J. H. 177
ROBERTS E. 203
ROBERTSON D. M. 145
ROBINS E. 18, 32
ROSENBLATT S. 172
SABOTKA H. 172
SACHS H. 297
SAIFER A. 218, 383
SAMORAJSKI 349
SAUNDERS P. R. 354
SCHOLEFIELD P. G. 177
SEGAR W. E. 150
SIMINOFF R. 354
SOURANDER P. 307
SPORN M. B. 62
SPYROPOULOS C. S. 185
STRECKER H. J. 50
SULKIN N. M. 231
THOMPSON R. H. S. 135
TOWER D. B. 80
VAN DER KLOOT W. G. 245
WAART D. DE 121
WEBSTER G. R. 135
WENDER M. 105
WISZNIOWSKA S. 109
WOLMAN M. 114
YABE T. 359
YAGI K. 304
YUWILER A. 73

Journal of NEUROCHEMISTRY

VOLUME 5, 1959/1960

OL.
5
9/60

Editorial Board

R. V. COXON (Oxford)
V. ENGELHARDT (Moscow)
A. ENGSTRÖM (Stockholm)
J. FOLCH-PI (Waverley, Mass.)
S. S. KETY (Bethesda, Md.)
E. KLENK (Cologne)
C. LONG (London)
A. POPE (Waverley, Mass.)
D. RICHTER (Carshalton, Surrey)
R. ROSSITER (London, Ont.)
W. M. SPERRY (New York)
M. VOGT (Cambridge)
H. WAELSCH (New York)

Advisory Board

G. B. ANSELL (Birmingham)
W. BARGMANN (Kiel)
J. BENOIT (Paris)
D. BOVET (Rome)
F. BRINK, JR. (New York)
J. N. CUMINGS (London)
R. M. C. DAWSON (Cambridge)
J. ELKES (Washington)
K. A. C. ELLIOTT (Montreal)
O. ERÄNKÖ (Helsinki)
V. ERSPAMER (Parma)
U. S. von EULER (Stockholm)
W. FELDBERG (London)
H. FERNÁNDEZ-MORÁN (Boston)
L. B. FLEXNER (Philadelphia, Pa.)
J. H. GADDUM (Cambridge)
R. W. GERARD (Ann Arbor, Mich.)
G. W. HARRIS (London)
C. HEBB (Cambridge)
H. HYDÉN (Göteborg)
Sir HANS KREBS (Oxford)
O. LINDBERG (Stockholm)
R. LORENTE de NÓ (New York)
O. H. LOWRY (St. Louis, Mo.)
H. McILWAIN (London)
A. v. MURALT (Berne)
A. V. PALLADIN (Kiev)
K. R. PORTER (New York)
J. H. QUASTEL (Montreal)
E. ROBERTS (Duarte, Calif.)
B. SCHARRER (New York)
F. O. SCHMITT (Cambridge, Mass.)
S. G. WAELSCH (New York)
P. WEISS (New York)

Pergamon Press

Oxford · London *New York · Paris*

JOURNAL OF NEUROCHEMISTRY

Editors:

R. V. COXON: *University Laboratory of Physiology, Oxford.*
V. ENGELHARDT: *The Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.*
A. ENGSTRÖM: *Department of Medical Physics, Karolinska Institutet, Stockholm, Sweden.*
J. FOLCH-PI: *McLean Hospital, Waverley, Mass., U.S.A.*
S. S. KETY: *Department of Health, Education and Welfare, National Institutes of Health, Bethesda* 14, *Md., U.S.A.*
E. KLENK: *Physiologisch-Chemisches Institut der Universität, Köln, Germany.*
C. LONG: *Royal College of Surgeons, Lincoln's Inn Fields, London W.C.*2.
A. POPE: *McLean Hospital, Waverley, Mass., U.S.A.*
D. RICHTER: *Neuropsychiatric Research Centre, Woodmansterne Road, Carshalton, Surrey.*
R. ROSSITER: *Department of Biochemistry, University of Western Ontario, London, Canada.*
W. M. SPERRY: *New York State Psychiatric Institute, New York* 32, *N.Y., U.S.A.*
M. VOGT: *Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge, England.*
H. WAELSCH: *New York State Psychiatric Institute, New York* 32, *N.Y., U.S.A.*

Publishing Offices: Headington Hill Hall, Oxford (Oxford 64881)

Issued in parts. Subscription per volume (A) £6 (*U.S.A.* $17.00) *including postage; (B) for individual subscribers who write direct to the publisher certifying that the journal is for their private use,* £3 10*s.* (*U.S.A.* $10.00) *including postage.*

Copyright © 1959/60, *Pergamon Press Ltd.*

VOL.
5
1959/6

AUTHOR'S INDEX FOR VOLUME 5

ADAMS C. W. M. and DAVISON A. N.: The form in which cholesterol occurs in the adult CNS 293
AGUILAR V. See BARKULIS, GEIGER, KAWAKITA and AGUILAR 339
AINSWORTH S. See PORTER and AINSWORTH 91
ALBERS W. See AXELROD, ALBERS and CLEMENTE 68
ANDO M. See YAGI, OZAWA, ANDO and NAGATSU 304
APRISON M. H., LUKENBILL A. and SEGAR W. E.: Sodium, potassium, chloride, and water content of six discrete parts of the mammalian brain 150
AXELROD J., ALBERS W. and CLEMENTE C. D.: Distribution of catechol-O-methyl transferase in the nervous system and other tissues 68
BALÁZS R. and LAGNADO J. R.: Glycolytic activity associated with rat brain mitochondria . 1
BARKULIS S. S., GEIGER A., KAWAKITA Y. and AGUILAR V.: A study on the incorporation of ^{14}C derived from glucose into the free amino acids of the brain cortex 339
BARKULIS S. S. See GEIGER, KAWAKITA and BARKULIS 323
BARKULIS S. S. See MARGOLIS, BARKULIS and GEIGER 379
BAXTER C. F., ROBERTS E. and EIDELBERG E.: γ-Aminobutyric acid and seizure susceptibility in areas of normal cat brain cortex 203
BONICHON A.: Développement de la cholinestérase au niveau du mésencéphale chez l'embryon de poulet 195
CANAL N. and MAFFEI-FACCIOLI A.: Reversal of the reserpine-induced depletion of brain serotonin by a monoamine oxidase inhibitor 99
CHAIKOFF I. L. See EMANUEL and CHAIKOFF 236
CHANLEY J. D. See ROSENBLATT, CHANLEY, SABOTKA and KAUFMAN 172
CLEMENTE C. D. See AXELROD, ALBERS and CLEMENTE 68
COHEN G. See HOCHSTEIN and COHEN 370
COHEN L. H. and NOELL W. K.: Glucose catabolism of rabbit retina before and after development of visual function 253
CURTI B. See PORCELLATI and CURTI 277

DAVIES R. K. See SPORN, DINGMAN, DEFALCO and DAVIES 62
DAVISON A. N. See ADAMS and DAVISON 293
DEFALCO A. See SPORN, DINGMAN, DEFALCO and DAVIES 62
DINGMAN W. See SPORN, DINGMAN, DEFALCO and DAVIES 62
DREWNOWSKA I. See KRAWCZYŃSKI, WISZNIOWSKA and DREWNOWSKA 109
EDSTRÖM J.-E.: Ribonucleic acid changes in the motoneurons of the frog during axon regeneration 43
EIDELBERG E. See BAXTER, ROBERTS and EIDELBERG 203
EIDUSON S. See GELLER, EIDUSON and YUWILER 73
EMANUEL C. F. and CHAIKOFF I. L.: Deoxyribonucleic acids of central nervous system, kidney, and spleen: a comparison of some chemical and physical properties 236
ERNSTING M. J. E., KAFOE W. F., NAUTA W. TH., OOSTERHUIS H. K. and DE WAART C.: Biochemical studies on psychotropic drugs—I. The effect of psychotropic drugs on γ-aminobutyric acid and glutamic acid in brain tissue 121
FRIEDE R. L.: Histochemical investigations on succinic dehydrogenase in the central nervous system—IV. A histochemical mapping of the cerebral cortex of the guinea pig 156
GÁRDOS G.: Potassium accumulation in guinea pig brain cortex slices 198
GELLER E., EIDUSON S. and YUWILER A.: Oxidation of *p*-phenylenediamine and adrenaline in enzymic and copper-catalysed reactions 73
GEIGER A., HORVATH N. and KAWAKITA Y.: The incorporation of ^{14}C derived from glucose into the proteins of the brain cortex, at rest and during activity 311
GEIGER A., KAWAKITA Y. and BARKULIS S. S.: Major pathways of glucose utilization in the brain in brain perfusion experiments *in vivo* and *in situ* 323
GEIGER A. See BARKULIS, GEIGER, KAWAKITA and AGUILAR 339
GEIGER A. See MARGOLIS, BARKULIS and GEIGER 379
GIUDITTA A. and STRECKER H. J.: Alternate pathways of pyridine nucleotide oxidation in cerebral tissue 50
HABA R. See NARUSE, KATO, KUROKAWA, HABA and YABE 359
HALLGREN B. and SOURANDER P.: The non-haemin iron in the cerebral cortex in Alzheimer's disease 307
HARRIS A. F. and SAIFER A.: The metachromasy of strandin 218
HARRIS A. F. and SAIFER A.: The interaction of strandin with basic proteins 383
HESS H. H. and POPE A.: Intralaminar distribution of cytochrome oxidase activity in human frontal isocortex 207
HESTRIN-LERNER S. See WOLMAN and HESTRIN-LERNER 114
HESTRIN-LERNER S. See MAGNES and HESTRIN-LERNER 128
HIEROWSKI M. See WENDER and HIEROWSKI 105
HOCHSTEIN P. and COHEN G.: The inhibitory effects of quinones and dihydric phenols on glucose metabolism in sub-cellular systems of brain 370
HORVATH N. See GEIGER, HORVATH and KAWAKITA 311
HUDSON A. J., QUASTEL J. H. and SCHOLEFIELD P. G.: The effects of heated snake venom on the phosphate metabolism of the rat spinal cord 177
KAFOE W. F. See ERNSTING, KAFOE, NAUTA, OOSTERHUIS and DE WAART 121
KATO M. See Naruse, KATO, KUROKAWA, HABA and YABE 359
KATO R.: Serotonin content of rat brain in relation to sex and age 202
KAUFMAN M. R. See ROSENBLATT, CHANLEY, SABOTKA and KAUFMAN 172
KAWAKITA Y. See BARKULIS, GEIGER, KAWAKITA and AGUILAR 339
KAWAKITA Y. See GEIGER, HORVATH and KAWAKITA 311
KAWAKITA Y. See GEIGER, KAWAKITA and BARKULIS 323
KRAWCZYŃSKI J., WISZNIOWSKA S. and DREWNOWSKA I.: Incorporation of radioactivity into proteins of the cerebrum, cerebellum and other organs, after the injection of ^{35}S in the form of sodium sulphate 109
KUROKAWA M.: Metabolic consequences of localized application of electrical pulses to sections of cerebral white matter 283
KUROKAWA M. See NARUSE, KATO, KUROKAWA, HABA and YABE 359
LAGNADO J. R. See BALÁZS and LAGNADO 1
LUKENBILL A. See APRISON, LUKENBILL and SEGAR 150

MAFFEI-FACCIOLO A. See CANAL and MAFFEI-FACCIOLO 99
MAGNES J. and HESTRIN-LERNER S.: Effect of 5-hydroxytryptophan and serotonin on glucose content and functional activity of the perfused cat brain 128
MARGOLIS R. U., BARKULIS S. S. and GEIGER A.: A comparison between the incorporation of ^{14}C from glucose into N-acetyl-L-aspartic acid and aspartic acid in brain perfusion experiments 379
MCARDLE B., THOMPSON R. H. S. and WEBSTER G. R.: The action of lysolecithin and of snake venom on whole-cell preparations of brain, muscle and liver 135
MCCAMAN R. E. and ROBINS E.: Quantitative biochemical studies of Wallerian degeneration in the peripheral and central nervous system—I. Chemical constituents 18
MCCAMAN R. E. and ROBINS E.: Quantitative biochemical studies of Wallerian degeneration in the peripheral and central nervous system—II. Twelve enzymes 32
NAGATSU T. See YAGI, OZAWA, ANDO and NAGATSU 304
NARUSE H., KATO M., KUROKAWA M., HABA R. and YABE T.: Metabolic defects in a convulsive strain of mouse 359
NAUTA W. TH. See ERNSTING, KAFOE, NAUTA, OOSTERHUIS and DE WAART 121
NOELL W. K. See COHEN and NOELL 253
OOSTERHUIS H. K. See ERNSTING, KAFOE, NAUTA, OOSTERHUIS and DE WAART 121
OZAWA T. See YAGI, OZAWA, ANDO and NAGATSU 304
PETERS E. L. and TOWER D. B.: Glutamic acid and glutamine metabolism in cerebral cortex after seizures induced by methionine sulphoximine 80
POPE A. See HESS and POPE 207
PORCELLATI G. and CURTI B.: Proteinase activity of peripheral nerves during Wallerian degeneration 277
PORTER H. and AINSWORTH S.: The isolation of the copper-containing protein cerebrocuprein I from normal human brain 91
QUASTEL J. H. See HUDSON, QUASTEL and SCHOLEFIELD 177
ROBERTS E. See BAXTER, ROBERTS and EIDELBERG 203
ROBERTSON D. M.: The paper electrophoretic distribution of soluble proteins in different regions of human brain 145
ROBINS E. See MCCAMAN and ROBINS 18
ROBINS E. See MCCAMAN and ROBINS 32
ROSENBLATT S., CHANLEY J. D., SABOTKA H. and KAUFMAN M. R.: Interrelationships between electroshock, the blood–brain barrier, and catecholamines 172
SABOTKA H. See ROSENBLATT, CHANLEY, SABOTKA and KAUFMAN 172
SACHS H.: Vasopressin biosynthesis—I. *In vivo* studies 297
SAIFER A. See HARRIS and SAIFER 218
SAIFER A. See HARRIS and SAIFER 383
SAMORAJSKI T.: The application of diphosphopyridine nucleotide diaphorase methods in a study of dorsal ganglia and spinal cord 349
SAUNDERS P. R. See SIMINOFF and SAUNDERS 354
SCHOLEFIELD P. G. See HUDSON, QUASTEL and SCHOLEFIELD 177
SEGAR W. E. See APRISON, LUKENBILL and SEGAR 150
SIMINOFF R. and SAUNDERS P. R.: Effect of morphine on the uptake and oxidation of glucose by rabbit brain 354
SOURANDER P. See HALLGREN and SOURANDER 307
SPORN M. B., DINGMAN W., DEFALCO A. and DAVIES R. K.: The synthesis of urea in the living rat brain 62
SPYROPOULOS C. S.: Cytoplasmic pH of nerve fibres 185
STRECKER H. J. See GIUDITTA and STRECKER 50
SULKIN N. M.: The distribution of mucopolysaccharides in the cytoplasm of vertebrate nerve cells 231
THOMPSON R. H. S. See MCARDLE, THOMPSON and WEBSTER 135
TOWER D. B. See PETERS and TOWER 80
VAN DER KLOOT W. G.: Factor S—a substance which excites crustacean muscle 245
WAART D. DE See ERNSTING, KAFOE, NAUTA, OOSTERHUIS and DE WAART 121
WEBSTER G. R. See MCARDLE, THOMPSON and WEBSTER 135

VOL.
5
1959/6

WENDER M. and HIEROWSKI M.: The concentration of electrolytes in the developing nervous system with special reference to the period of myelination 105
WISZNIOWSKA S. See KRAWCZYŃSKI, WISZNIOWSKA and DREWNOWSKA 109
WOLMAN M. and HESTRIN-LERNER S.: A histochemical contribution to the study of the molecular morphology of myelin sheath 114
YABE T. See NARUSE, KATO, KUROKAWA, HABA and YABE 359
YAGI K., OZAWA T., ANDO M. and NAGATSU T.: The effect of flavin adenine dinucleotide on the electroencephalogram modified by chlorpromazine 304
YUWILER A. See GELLER, EIDUSON and YUWILER 73

OL.
5
9/60

SUBJECT INDEX FOR VOLUME 5

Adrenaline and *p*-phenylene-diamine, oxidation of, in enzymic and copper-catalysed reactions . 73
Alzheimer's disease, non-haemin iron in cerebral cortex in 307
Amino acids, free, of brain cortex, a study on incorporation of ^{14}C derived from glucose into . 339
Aspartic acid and N-acetyl-L-aspartic acid, a comparison between incorporation of ^{14}C from glucose into, in brain perfusion experiments 379
Axon regeneration, ribonucleic acid changes in motoneurons of frog during 43
Basic proteins, interaction of strandin with 383
Biochemical studies, quantitative, of Wallerian degeneration in the peripheral and central nervous system
I—Chemical constituents 18
II—Twelve enzymes 32
Biosynthesis, vasopressin
I—*In vivo* studies 297
Blood–brain barrier, electroshock, and catecholamines, interrelationships between . . 172
Brain, cat, perfused, effect of 5-hydroxytryptophan and serotonin on glucose content and functional activity of 128
Brain cortex, cat, normal, γ-aminobutyric acid and seizure susceptibility in areas of . . 203
Brain cortex, incorporation of ^{14}C derived from glucose into proteins of, at rest and during activity 311
Brain cortex slices, guinea pig, potassium accumulation in 198
Brain cortex, a study on incorporation of ^{14}C derived from glucose into free amino acids of . 339
Brain, human, normal, isolation of copper-containing protein cerebrocuprein I from . . 91
Brain, human, paper electrophoretic distribution of soluble proteins of different regions of . 145
Brain, inhibitory effects of quinones and dihydric phenols on glucose metabolism in sub-cellular systems of 370
Brain, major pathways of glucose utilization in, in brain perfusion experiments *in vivo* and *in situ* 323
Brain, mammalian, sodium, potassium, chloride and water content of six discrete parts of . 150
Brain mitochondria, rat, glycolytic activity associated with 1
Brain, muscle and liver, action of lysolecithin and of snake venom on whole cell preparations of 135
Brain perfusion experiments, a comparison between incorporation from ^{14}C from glucose into N-acetyl-L-aspartic acid and aspartic acid in 379
Brain, rabbit, effect of morphine on uptake and oxidation of glucose by 354
Brain, rat, living, synthesis of urea in 62
Brain, rat, serotonin content of, in relation to sex and age 202
Brain serotonin, reversal of reserpine-induced depletion of, by a monoamine oxidase inhibitor . 99
Brain tissue, effect of psychotropic drugs on γ-aminobutyric acid and glutamic acid in. Biochemical studies on psychotropic drugs—I 121
Catabolism, glucose, of rabbit retina before and after development of visual function . . 253
Catechol-O-methyl transferase distribution in the nervous system and other tissues . . 68
Catecholamines, electroshock, and blood–brain barrier, interrelationships between . . 172
Central nervous system, histochemical investigations on succinic dehydrogenase in—IV. Histochemical mapping of cerebral cortex of guinea pig 156
Central nervous system, kidney, and spleen, deoxyribonucleic acids of: a comparison of some chemical and physical properties 236
Cerebral cortex, glutamic acid and glutamine metabolism in, after seizures induced by methionine sulphoximine 80
Cerebral cortex, guinea pig, histochemical mapping of. Histochemical investigations on succinic dehydrogenase in central nervous system—IV 156
Cerebral cortex, non-haemin iron in, in Alzheimer's disease 307
Cerebral tissue, alternate pathways of pyridine nucleotide oxidation in 50
Cerebral white matter, metabolic consequences of localized application of electrical pulses to sections of 283

VOL.
5
1959/6

Cerebrum, cerebellum and other organs, incorporation of radioactivity into proteins of, after injection of ^{35}S in form of sodium sulphate 109
Chloride, sodium, potassium and water content of six discrete parts of mammalian brain . . 150
Chlorpromazine, effect of flavin adenine dinucleotide on the electroencephalogram modified by 304
Cholesterol, form in which it occurs in adult CNS 293
Cholinesterase, development of, in mesencephalon of chick embryo 195
Convulsive strain of mouse, metabolic defect in 359
Copper-catalysed and enzymic reactions, oxidation of *p*-phenylenediamine and adrenaline in . 73
Copper-containing protein cerebrocuprein I, isolation from normal human brain 91
Cortex, brain, cat, normal, γ-aminobutyric acid and seizure susceptibility in areas of . . . 203
Cortex, brain, incorportaion of ^{14}C derived from glucose into proteins of, at rest and during activity 311
Cortex, brain, a study on incorporation of ^{14}C derived from glucose into free amino acids of 339
Cortex, cerebral, glutamic acid and glutamine metabolism in, after seizures induced by methionine sulphoximine 80
Cortex, cerebral, guinea pig, histochemical mapping of. Histochemical investigations on succinic dehydrogenase in central nervous system—IV 156
Cortex, cerebral, non-haemin iron in, in Alzheimer's disease 307
Cortex slices, brain, guinea pig, potassium accumulation in 198
Crustacean muscle, Factor S—a substance which excites 245
Cytochrome oxidase activity in human frontal cortex, intralaminar distribution of 207
Cytoplasm of vertebrate nerve cells, distribution of mucopolysaccharides in 231
Cytoplasmic pH of nerve fibres 185
Defects, metabolic, in a convulsive strain of mouse 359
Degeneration, Wallerian, proteinase activity of peripheral nerves during 277
Degeneration, Wallerian, quantitative biochemical studies of, in the peripheral and central nervous systems
I—Chemical constituents 18
II—Twelve enzymes 32
Dehydrogenase, succinic, histochemical investigations on, in central nervous system—IV. Histochemical mapping of cerebral cortex of guinea pig 156
Deoxyribonucleic acids of central nervous system, kidney, and spleen: a comparison of some chemical and physical properties 236
Developing nervous system, concentration of electrolytes in, with special reference to period of myelination 105
Development of cholinesterase in mesencephalon of chick embryo 195
Development of visual function, glucose catabolism of rabbit retina before and after . . 253
Diaphorase, diphosphopyridine nucleotide, methods, application of, in a study of dorsal ganglia and spinal cord 349
Dihydric phenols and quinones, inhibitory effects of, on glucose metabolism in sub-cellular systems of brain 370
Diphosphopyridine nucleotide diaphorase methods, application of, in a study of dorsal ganglia and spinal cord 349
Distribution of catechol-O-methyl transferase in nervous system and other tissues . . . 68
Distribution of mucopolysaccharides in the cytoplasm of vertebrate nerve cells . . . 231
Distribution, paper electrophoretic, of soluble proteins in different regions of human brain . 145
Dorsal ganglia and spinal cord, application of diphosphopyridine nucleotide diaphorase methods in a study of 349
Drugs, psychotropic, biochemical studies—I. Effect of psychotropic drugs on γ-aminobutyric acid and glutamic acid in brain tissue 121
Electrical pulses, metabolic consequences of localized application of, to sections of cerebral white matter 283
Electroencephalogram modified by chlorpromazine, effect of flavin adenine dinucleotide on . 304
Electrolytes, concentration of, in developing nervous system with special reference to period of myelination 105
Electrophoretic, paper, distribution of soluble proteins in different regions of human brain . 145
Electroshock, blood–brain barrier, and catecholamines, interrelationships between . . 172

Embryo, chick, development of cholinesterase in mesencephalon of 195
Enzymic and copper-catalysed reactions, oxidation of *p*-phenylenediamine and adrenaline in . 73
Factor S—a substance which excites crustacean muscle 245
Flavin adenine dinucleotide, effect of, on electroencephalogram modified by chlorpromazine . 304
Free amino acids of brain cortex, a study on incorporation of ^{14}C derived from glucose into . 339
Functional activity and glucose content of perfused cat brain, effect of 5-hydroxytryptophan and serotonin on 128
Gamma-aminobutyric acid and glutamic acid in brain tissue, effect of psychotropic drugs on. Biochemical studies on psychotropic drugs—I 121
Gamma-aminobutyric acid and seizure susceptibility in areas of normal cat brain cortex . . 203
Ganglia, dorsal, and spinal cord, application of diphosphopyridine nucleotide diaphorase methods in a study of 349
Glucose catabolism of rabbit retina before and after development of visual function . . 253
Glucose, a comparison between incorporation of ^{14}C from, into N-acetyl-L-aspartic acid and aspartic acid in brain perfusion experiments 379
Glucose content and functional activity of perfused cat brain, effect of -5hydroxytryptophan and serotonin on 128
Glucose, effect of morphine on uptake and oxidation of, by rabbit brain 354
Glucose, incorporation of ^{14}C derived from, into free amino acids of brain cortex, a study . . 323
Glucose, incorporation of ^{14}C derived from, into proteins of brain cortex, at rest and during activity 311
Glucose metabolism, inhibitory effects of quinones and dihydric phenols on, in sub-cellular systems of brain 370
Glucose utilization, major pathways of, in brain in brain perfusion experiments *in vivo* and *in situ* 323
Glutamic acid and γ-aminobutyric acid in brain tissue, effect of psychotropic drugs on. Biochemical studies on psychotropic drugs—I 121
Glutamic acid and glutamine metabolism in cerebral cortex after seizures induced by methionine sulphoximine 80
Glutamine and glutamic acid metabolism in cerebral cortex after seizures induced by methionine suphoximine 80
Glycolytic activity associated with rat brain mitochondria 1
Histochemical contribution to study of molecular morphology of meylin sheath . . . 114
Histochemical investigations on succinic dehydrogenase in central nervous system—IV. Histochemical mapping of cerebral cortex of guinea pig 156
Human brain, normal isolation of copper-containing protein cerebrocuprein I from . . 91
Human brain, paper electrophoretic distribution of soluble proteins of different regions of . 145
Human frontal isocortex, intralaminar distribution of cytochrome oxidase activity in . . 207
5-Hydroxytryptophan and serotonin, effect of, on glucose content and functional activity of perfused cat brain 128
Incorporation of ^{14}C derived from glucose into free amino acids of brain cortex, a study on . 323
Incorporation of ^{14}C derived from glucose into proteins of brain cortex, at rest and during activity 311
Incorporation of ^{14}C from glucose into N-acetyl-L-aspartic acid and aspartic acid in brain perfusion experiments, a comparison 379
Incorporation of radioactivity into proteins of cerebrum, cerebellum and other organs, after injection of ^{35}S in form of sodium sulphate 109
Iron, non-haemin, in cerebral cortex in Alzheimer's disease 307
Isocortex, frontal, human, intralaminar distribution of cytochrome oxidase activity in . . 207
Kidney, spleen, and central nervous system, deoxyribonucleic acids of: a comparison of some chemical and physical properties 236
Lysolecithin, and snake venom, action of, on whole-cell preparations of brain, muscle and liver 135
Mesencephalon of chick embryo, development of cholinesterase in 195
Metabolic consequences of localized application of electrical pulses to sections of cerebral white matter 283
Metabolic defects in a convulsive strain of mouse 359

VOL.
5
1959/6

Metabolism, glucose, inhibitory effects of quinones and dihydric phenols on, in sub-cellular systems of brain 370
Metabolism, glutamic acid and glutamine, in cerebral cortex after seizures induced by methionine sulphoximine 80
Metabolism, phosphate, of rat spinal cord, effects of heated snake venom on 177
Metachromasy of strandin 218
Methionine sulphoximine, glutamic acid and glutamine metabolism in cerebral cortex after seizures induced by 80
Mitochondria, rat brain, glycolytic activity associated with 1
Monoamine oxidase inhibitor, reversal of reserpine-induced depletion of brain serotonin by . 99
Morphine, effect of, on uptake and oxidation of glucose by rabbit brain 354
Motoneurons, frog, ribonucleic acid changes in, during axon regeneration 43
Mucopolysaccharides, distribution of, in cytoplasm of vertebrate nerve cells 231
Muscle, brain and liver, action of lysolecithin and of snake venom on whole-cell preparations of 135
Muscle, crustacean, Factor S—a substance which excites 245
Myelin sheath, histochemical contribution to study of molecular morphology of 114
Myelination, concentration of electrolytes in developing nervous system with special reference to period of 105
Nerve cells, vertebrate, distribution of mucopolysaccharides in cytoplasm of 231
Nerve fibres, cytoplasmic pH of 185
Nerves, peripheral, proteinase activity of, during Wallerian degeneration 277
Nervous system, developing, concentration of electrolytes in, with special reference to period of myelination 105
Nervous system and other tissues, distribution of catechol-O-methyl transferase in . . 68
Nucleotide, pyridine, oxidation in cerebral tissue, alternate pathways of 50
Oxidation of *p*-phenylenediamine and adrenaline in enzymic and copper-catalysed reactions . 73
Oxidation and uptake of glucose by rabbit brain, effect of morphine on 354
pH, cytoplasmic, of nerve fibres 185
Pathways, major, of glucose utilization in brain in brain perfusion experiments *in vivo* and *in situ* 323
Pathways, alternate, of pyridine nucleotide oxidation in cerebral tissue 50
Perfusion, brain, experiments *in vivo* and *in situ*, major pathways of glucose utilization in brain in 323
Peripheral and central nervous systems, quantitative biochemical studies of Wallerian degeneration in
I—Chemical constituents 18
II—Twelve enzymes 32
Peripheral nerves, proteinase activity of, during Wallerian degeneration 277
Phenols, dihydric, and quinones, inhibitory effects of, on glucose metabolism in sub-cellular systems of brain 370
p-Phenylenediamine and adrenaline, oxidation of, in enzymic and copper-catalysed reactions . 73
Phosphate metabolism of rat spinal cord, effects of heated snake venom on 177
Potassium accumulation in guinea pig brain cortex slices 198
Potassium, sodium, chloride and water content of six discrete parts of mammalian brain . . 150
Protein, cerebrocuprein I, copper-containing, isolation from normal human brain . . . 91
Proteinase activity of peripheral nerves during Wallerian degeneration 277
Proteins, basic, interaction of strandin with 383
Proteins of brain cortex, incorporation of ^{14}C derived from glucose into, at rest and during activity 311
Proteins of cerebrum, cerebellum and other organs, incorporation of radioactivity into, after injection of ^{35}S in form of sodium sulphate 109
Proteins, soluble, in different regions of brain, paper electrophoretic distribution of . . 145
Psychotropic drugs, biochemical studies on—I. Effect of psychotropic drugs on γ-aminobutyric acid and glutamic acid in brain tissue 121
Pulses, electrical, metabolic consequences of localized application of, to sections of cerebral white matter 283
Pyridine nucleotide oxidation in cerebral tissue, alternate pathways of 50

Quinones and dihydric phenols, inhibitory effects of, on glucose metabolism in sub-cellular systems of brain 370
Radioactivity, incorporation of, into proteins of cerebrum, cerebellum and other organs, after injection of ^{35}S in form of sodium sulphate 109
Regeneration, axon, ribonucleic acid changes in motoneurons of frog during 43
Reserpine-induced depletion of brain serotonin, reversal by a monoamine oxidase inhibitor . 99
Retina, rabbit, glucose catabolism of, berore and after development of visual function . . 253
Ribonucleic acid changes in the motoneurons of the frog during axon regeneration . . 43
^{35}S, incorporation of radioactivity into proteins of cerebrum, cerebellum and other organs after injection of, in form of sodium sulphate 109
Seizure susceptibility and γ-aminobutyric acid in areas of normal cat brain cortex . . 203
Seizures induced by methionine sulphoximine, glutamic acid and glutamine metabolism in cerebral cortex after 80
Serotonin, brain, reversal of reserpine-induced depletion of, by a monoamine oxidase inhibitor 99
Serotonin content of rat brain in relation to sex and age 202
Serotonin and 5-hydroxytryptophan, effect of, on glucose content and functional activity of perfused cat brain 128
Snake venom, heated, effects of, on phosphate metabolism of rat spinal cord 177
Snake venom, and lysolecithin, action of, on whole-cell preparations of brain, muscle and liver 135
Sodium, potassium, chloride and water content of six discrete parts of mammalian brain . . 150
Spinal cord and dorsal ganglia, application of diphosphopyridine nucleotide diaphorase methods in a study of 349
Spinal cord, rat, effects of heated snake venom on phosphate metabolism of 177
Spleen, kidney, and central nervous system, deoxyribonucleic acids of: a comparison of some chemical and physical properties 236
Strandin, interaction of, with basic proteins 383
Strandin, metachromasy of 218
Succinic dehydrogenase, histochemical investigations on, in central nervous system—IV. Histochemical mapping of cerebral cortex of guinea pig 156
Synthesis of urea in living rat brain 62
Tissue, cerebral, alternate pathways of pyridine nucleotide oxidation in 50
Transferase, catechol-O-methyl, distribution in nervous system and other tissues . . . 68
Tissues, distribution of catechol-O-methyl transferase in nervous system and other tissues . 68
Uptake and oxidation of glucose by rabbit brain, effect of morphine on 354
Urea synthesis in living rat brain 62
Vasopressin biosynthesis—I. *In vivo* studies 297
Visual function, glucose catabolism of rabbit retina before and after development of . . 253
Wallerian degeneration, proteinase activity of peripheral nerves during 277
Wallerian degeneration, quantitative biochemical studies of, in the peripheral and central nervous systems
I—Chemical constituents 18
II—Twelve enzymes 32
Water, sodium, potassium and chloride content of six discrete parts of mammalian brain . . 150
White matter, cerebral, metabolic consequences of localized application of electrical pulses to sections of 283

Journal of Neurochemistry, 1959, Vol. 5, pp. 1 to 17. Pergamon Press Ltd. Printed in Northern Ireland

GLYCOLYTIC ACTIVITY ASSOCIATED WITH RAT BRAIN MITOCHONDRIA

R. BALÁZS and J. R. LAGNADO*
Medical Research Council, Neuropsychiatric Research Unit,
Whitchurch Hospital, Cardiff, Wales

(*Received* 14 *February* 1959)

IT HAS generally been assumed that glycolytic activity is confined to the soluble fraction of tissue homogenates: this has been found for liver and other tissues (LEHNINGER, 1951). HESSELBACH and DU BUY (1953) reported however that in mouse brain homogenates as much as 20 per cent of the glycolytic activity is associated with the mitochondrial fraction, and glycolytic activity has also been found in mitochondria prepared from the rat brain (GALLAGHER, JUDAH and REES, 1956; STRECKER, 1957; BALÁZS and RICHTER, 1958; BALÁZS, 1959). On the other hand BRODY and BAIN (1952) found no glycolytic activity in their preparations of rat brain mitochondria, and a similar negative finding was reported by ALDRIDGE (1957). The reasons for this discrepancy in the properties reported for brain mitochondria are not clear. The glycolytic activity in the mitochondria found by some workers might be attributed to contamination by other tissue fractions or adsorption of glycolytic enzymes on the mitochondria. In this connexion the inclusion in this fraction of the 'light particles' or 'fluffy layer' might be relevant. Alternatively the failure of some workers to find glycolytic activity might be due to the removal of cofactors needed for glycolysis.

The purpose of the present work was to obtain further information about the glycolytic activity associated with brain mitochondria. The effects of different fractionation and washing procedures were studied, and the activities of individual glycolytic enzymes were measured in mitochondria prepared by different methods.

EXPERIMENTAL

The Preparation of Brain Homogenate Fractions

(a) '*Sucrose mitochondria.*' Mitochondria were prepared from brains of Wistar Albino rats (120–230 g) by a method similar to that originally used by BRODY and BAIN (1952) (Fraction-1-R_3). The freshly excised brains were first homogenized in a NIRD blender (Nelco Ltd., similar to a Waring blender) for 1 min in a small volume of cold 0·25 M-sucrose containing 0·025 M-NAA,† and then once more after the addition of more sucrose solution in order to give a 10% (w/v) suspension. The suspension was centrifuged twice in a MSE 'Super-Speed 20' centrifuge for 10 min at 1500 g to remove nuclei and cellular debris. The resulting supernatant was then centrifuged for 10 min at 18,000 g to sediment the mitochondria, which were washed once with the same sucrose solution. The mitochondrial sediment was suspended in a salt solution and diluted to the desired volume. The composition of the salt solution was as follows: 100 ml 0·154 M-NaCl, 1·0 ml 0·154 M-$MgSO_4$, 5·25 ml 0·5 M-NAA, 10 ml 0·1 M-K-phosphate buffer, pH 7·4. Replacement of NaCl by KCl did not alter the results. The entire procedure was carried out at 0–5° and the mitochondria were used within 30 min of preparation. (Lactic dehydrogenase activity was usually measured within 24 hr,

* Kathleen Schlesinger Fellow.

† *Abbreviations:* adenosinetriphosphate, ATP; 5′-adenylic acid, AMP; diphosphopyridinenucleotide, DPN; 2-amino-2-hydroxy-methylpropane-1:3-diol, tris; ethylenediaminetetraacetic acid, EDTA; nicotinamide, NAA; fructose diphosphate, HDP; triosephosphate dehydrogenase, TP-dehydrogenase.

during which time no loss in activity occurred.) Any deviations from the above procedure are specified in the text.

(b) '*Saline-mitochondria.*' Brains were suspended in a modified Krebs–Ringer phosphate medium (containing 0·025 M-NAA) in a NIRD blender to give a final suspension of 10% (w/v). The composition of the medium was as follows: 100 parts 0·154 M-NaCl, 4 parts 0·154 M-KCl, 1 part 0·154 M-$MgSO_4$, 11 parts 0·1 M-K-phosphate buffer, pH 7·1. The suspension was centrifuged at 800 g for 10 min and the supernatant fluid recentrifuged at 18,000 g for 10 min. The residue obtained was washed twice and suspended in a small volume of the same medium. This preparation will be designated as 'saline-mitochondria.'

Glycolytic Assays

(a) *Reaction mixture.* The *standard medium* contained 0·005–0·01 M-glucose, 0·006 M-$MgSO_4$ 0·01 M-K-phosphate buffer (pH 7·4), 0·01 M-tris buffer (neutralized with HCl to pH 7·4), 0·002 M-EDTA (neutralized with KOH to pH 7·4) and 0·025 M-NAA (final concentrations given). To this *standard medium* various compounds were added as specified in the experimental part. Tricarboxylic acid cycle intermediates and the adenine nucleotides were neutralized with KOH before addition. The final volume in the flasks was 1·5 ml.

(b) *Incubation.* Incubations were carried out in conventional Warburg vessels at 37·5°. For the anaerobic experiments the vessels were gassed with nitrogen (freed from traces of oxygen by passage through a heated copper tube), while the 'aerobic' samples were run in air. The incubation time was usually 30–60 min. The reaction was stopped in most cases with perchloric acid (final concentration 0·3 M) which was tipped in from the side-arm. In the experiments where glucose was also determined, a sample of the flask content was pipetted directly into cold 0·2 N-$ZnSO_4$ and 0·3 N-$Ba(OH)_2$ was added 1–5 min later.

When the oxygen consumption was measured by the Warburg method (UMBREIT, BURRIS and STAUFFER, 1945), the vessels contained 0·1 ml of 20% KOH in the centre well. Temperature equilibration was carried out with open stop-cocks for 6–10 min and respiration values were extrapolated for this pre incubation period.

Analytical Methods

Glucose and lactate were determined by the methods of NELSON (1944) and BARKER and SUMMERSON (1941), respectively. A fairly high concentration of triosephosphates could be expected in some experiments. Since these intermediates are alkali-labile, and especially sensitive to alkaline earth cations (MEYERHOF and LOHMANN, 1934), special precautions were taken in the alkaline precipitation used in the lactate determination. Pure triosephosphates are hydrolysed to the extent of about 40 per cent at 25° in 30 min, giving rise to products which are determined as lactate by the method used. However, less than 5 per cent hydrolysis occurred at 0°. Therefore the samples were usually precipitated with the $CuSO_4$–CaO mixture at 0° and kept in an ice-bath for 30 min. (The rest of the procedure was carried out without further modification.)

Protein determinations were made according to the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951). A standard curve was run using bovine serum albumin.

Enzyme Assays

Hexokinase activity was assayed by measuring glucose disappearance in a reaction mixture containing the *standard medium* (0·005 M-glucose) supplemented with 0·0066 M-α-ketoglutarate, 0·002 M-ATP, 0·00033 M-DPN and 0·025 M-NaF (total volume, 1·0 ml; final concentrations given). This reaction mixture was used for purposes of comparison with glycolytic measurements, but very similar results were obtained when hexokinase activity was assayed in a mixture similar to that used by LONG (1952). Reaction was initiated by addition of enzyme (supernatant or homogenate preparations contained 100–300 μg protein; mitochondrial preparations contained 500–1500 μg protein) and terminated by precipitation with the zinc–barium mixture of SOMOGYI (1945). The incubation was carried out aerobically at 37·5° (unless otherwise stated) without shaking in 15 × 100 mm tubes for 10–20 min, and a zero-time control was included in all assays. Hexokinase was routinely measured at two levels of tissue concentration and at several time intervals. Under these conditions linearity was usually obtained with regard to time and tissue concentration (Table 1). Specific activities were calculated from the linear portions of the curves.

VOL. 5 1959/6

As previously reported by LONG (1952), fluoride was essential for the measurement of hexokinase (Table 1). Under the experimental conditions used, ATP-ase activity was inhibited by about 75 per cent. Hexokinase activity was very similar in brain homogenates prepared in sucrose or Krebs–Ringer–phosphate medium (Table 1). The pH curves for hexokinase of 'sucrose mitochondria' or of homogenates were very similar to those reported by CRANE and SOLS (1953).

Aldolase. The method of SIBLEY and LEHNINGER (1949) as modified by DOUNCE, BARNETT and BEYER (1950) was used to measure aldolase activity. The reaction mixtures contained the following

TABLE 1.—HEXOKINASE ACTIVITY IN RAT BRAIN HOMOGENATE AND MITOCHONDRIAL PREPARATIONS

Homogenate and mitochondrial preparations were obtained as specified in Experimental. *Incubation medium* as in Table 4, supplemented with 0·025 M-NaF. Incubations carried out in air at 37·5.

Expt. no.	Homogenate (mg-equivalent fresh tissue)	Time (min)	Glucose consumed (μmoles/tube)	Expt. no.	Mitochondria (mg-equivalent fresh tissue)	Time (min)	Glucose consumed (μmoles/tube)
1	10	30	0·83	4	20	10	0·32
	20	30	1·65		20	20	0·68
	30	30	2·20		40	13	0·60
	30*	30	0·78		80	13	1·10
2	10	10	0·10				
	10	20	0·22				
	10	30	0·33				
3a†	20	15	0·96				
3b	20	15	1·24				

* NaF omitted.

† Unfractionated homogenates prepared in sucrose (Expt. 3a) and in Krebs–Ringer–phosphate medium (Expt. 3b).

constituents: 0·005 M-Na_2-HDP, 0·04 M-collidine buffer, pH 7·4, 0·002 M-iodoacetic acid, 0·056 M-hydrazine sulphate, enzyme and water to give final volume of 1·25 ml. The final pH was adjusted to 7·1. Incubations were carried out at 37·5° and enzyme activity was assayed twice and at two levels of tissue concentration. Specific activity values were calculated from the linear portions of the curves. The routine assays were carried out for 10 and 20 min using amounts of tissue containing 150–350 μg protein for mitochondrial suspensions, and 25–50 μg protein for other fractions assayed. Under these conditions, linearity was obtained with regard to time and tissue concentration.

Aldolase activity of the brain mitochondrial suspensions was higher in tris than in collidine buffer at all pH values tested between pH 6·4 and 9·0 (about 50 per cent higher at pH 7·1). One small peak was observed in tris buffer at pH 7·1–7·3, while no peak was observed using collidine buffer. A second peak in tris buffer at alkaline pH values was due to non-enzymic chromogen formation: controls omitting enzyme were therefore run between pH 8 and 9 (DOUNCE *et al.*, 1950). Mitochondrial aldolase activity increased with temperature between 27·5° and 47·5°. Taking the activity at 37·5° as 100 per cent, the activity observed at 27·5° and 47·5° was 65 and 195 per cent respectively.

Triosephosphate dehydrogenase could not be assayed by the spectrophotometric methods based on DPN reduction, since in the crude tissue preparations large interferences could be expected from the action of other pyridine nucleotide-linked enzymes. The principle of the assay (MEYERHOF and KIESSLING, 1935) is that when the incubation is carried out using HDP as substrate in the presence of fluoride and arsenate, reduction of added pyruvate is a measure of TP-dehydrogenase activity when both aldolase and lactic dehydrogenase are present in excess (as in the case in brain homogenate

fractions tested, cf. Table 6). The validity of the assay was checked by comparing lactate and phosphoglycerate formation. As shown in Table 2, the values obtained for phosphoglycerate (measured by the method of MEYERHOF and SCHULTZ, 1938) and for the lactate were in sufficiently close agreement to justify this assay.

The incubation medium used is given in Table 2. Incubations were routinely carried out anaerobically in Warburg flasks at 37·5° and stopped with perchloric acid under the conditions used to measure glycolytic activity. Lactate estimations were carried out in the usual manner.

TABLE 2.—TRIOSEPHOSPHATE DEHYDROGENASE ACTIVITY IN RAT BRAIN MITOCHONDRIAL SUSPENSIONS

Incubation medium. 0·006–0·011 M-HDP, 0·0037 M-α-ketoglutarate, 0·002 M-ATP or AMP, 0·00033 M-DPN, 0·01 M-NaF, 0·003–0·012 M-pyruvate, 0·008 M-Na_2HAsO_4, 0·004 M-K-phosphate buffer, pH 7·4, 0·01 M-tris buffer, pH 7·4, 0·006 M-$MgSO_4$, 0·002 M-EDTA, 0·025 M-NAA. Mitochondria, mg-equivalent fresh brain/vessel: Expt. 1, 4, 250; Expt. 2, 3, 200; Expt. 5, 300. Total vol: 1·5–3 ml. Incubation: 40 min at 37·5°. 3-Phosphoglyceric acid determined by the method of MEYERHOF and SCHULZ (1938). Activity expressed as μmoles phosphoglycerate or lactate formed/vessel/40 min.

Expt. no.	3-Phosphoglycerate			Lactate		Aerobic inhibition* (%)
	Air	N_2	Aerobic inhibition* (%)	Air	N_2	
1	3·37	4·80	30	3·18	6·0	47
2	3·0	4·77	27	2·75	4·05	32
3	2·76	4·0	31	2·35	3·50	30
4	3·25	5·90	45	4·55	6·50	30
5	2·30	7·15	68	2·10	9·0	77

* Percentage inhibition of aerobic lactate (or phosphoglycerate) production.

$$\frac{\text{lactate}_{N_2} - \text{lactate}_{air}}{\text{lactate}_{N_2}} \times 100$$

Lactic dehydrogenase. This enzyme was assayed by the method of NEILANDS (1955). The test reaction mixture contained the following constituents (in a final volume of 2·57 ml): 0·00078 M-DPN, 0·0195 M-lactate, 0·093 M-glycine–NaOH buffer, pH 10. Two controls were run, one omitting the lactate, the other omitting the tissue. DPN reduction was followed at 340 mμ in a Hilger and Watts (Uvispek) spectrophotometer for up to 10 min, readings being taken every 15–30 sec during the first few minutes after a brief stirring period following addition of the enzyme preparation (mitochondrial suspension contained about 100 μg protein; homogenate and supernatant, about 50 μg protein). It was found that some decrease in optical density at 340 mμ occurred when mitochondrial and homogenate preparations were assayed, which was probably due to the reoxidation of endogenous pyridine nucleotides. Experimental values were corrected accordingly. It should be pointed out that specific activity values for lactic dehydrogenase activity were far below those which would be observed if the enzyme were assayed under conditions used to measure glycolysis (in the directi on pyruvate → lactate at 37·5° at pH 7·4).

RESULTS

Glucose utilization in brain mitochondria

Rat brain mitochondria prepared in 0·25 M-sucrose (containing 0·025 M-NAA) utilized glucose as substrate (Fig. 1). Furthermore, the respiration rate maintained

VOL. 5 1959/6

by Krebs cycle intermediates was slightly stimulated and remained linear in the presence of glucose. In this case, it is probable that glucose acted as phosphate-acceptor (LARDY, 1955). Added glucose is glycolysed in this preparation, as shown by the formation of lactate and the appearance of the usual glycolytic intermediates (BALÁZS, 1959). Glycolysis is inhibited under aerobic conditions. Factors determining the rates of glycolysis and the magnitude of the aerobic inhibitions are summarized in Table 3. The addition of ATP and of DPN stimulates glycolysis (Expt. 3). Their

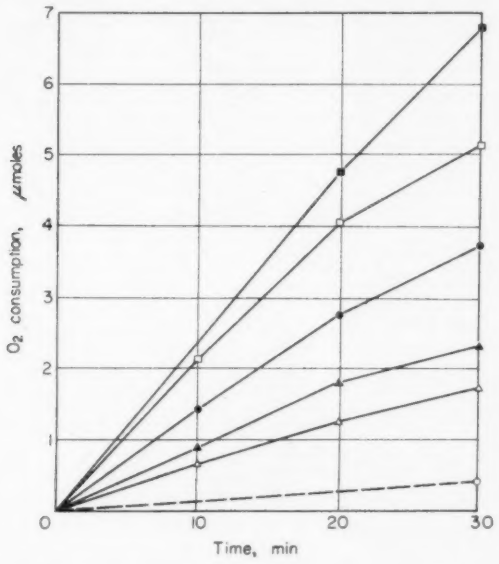


FIG. 1.—The effects of Krebs cycle intermediates and glucose on the respiration of rat brain mitochondria. Reaction mixture: 0·006 M-$MgSO_4$; 0·01 M-K-phosphate buffer, pH 7·4; 0·01 M-tris buffer, pH 7·4; 0·002 M-EDTA; 0·025 M-NAA; 0·002 M-ATP; 0·00033 M-DPN. Additions: ○ none; △ 0·01 M-glucose; ▲ 0·01 M-glucose, 0·003 M-pyruvate; ● 0·01 M-glucose, 0·002 M-L-malate; □ 0·003 M-pyruvate, 0·002 M-L-malate; ■ 0·01 M-glucose, 0·003 M-pyruvate, 0·002 M-L-malate. Mitochondria: 250 mg-equivalent fresh brain. Total volume: 1·60 ml.

combined addition is necessary to obtain maximal rates of respiration and of aerobic glucose consumption. The aerobic inhibition of lactate formation can only be relieved by the combined addition of ATP and DPN. Krebs cycle intermediates show no effect on glycolysis, respiration, or on the aerobic inhibition of lactate formation unless added in conjunction with ATP and DPN, in which case the inhibition is maximally relieved (Expt. 1, 2, 4).

Comparison of the respiration and of the glycolytic activity associated with mitochondria prepared from Potter–Elvehjem homogenates and from NIRD blends

Pooled rat brains were divided into two portions, one of which was suspended by means of a NIRD Blender (cf. Experimental), and the other by homogenizing for 2 min by hand in a glass tube fitted with a Perspex pestle (0·02 in. clearance) (similar to Potter–Elvehjem homogenizer), to give final suspensions of 10% (w/v) in 0·25

M-sucrose. It was found that glycolytic activity was similar for mitochondria prepared from both types of suspensions, as judged by lactate production and aerobic glucose consumption (Table 4). The percentage aerobic inhibition values, as well as the specific activities of individual glycolytic enzymes tested, were the same in both types

TABLE 3.—EFFECT OF COFACTORS AND KREBS CYCLE INTERMEDIATES ON GLUCOSE METABOLISM IN RAT BRAIN MITOCHONDRIAL PREPARATIONS

System: *standard medium* (cf. Experimental). Additives: Krebs cycle intermediates and cofactors (0·002 M-ATP, 0·00033 M-DPN) as indicated. Final volume: 1·5 ml. Temperature: 37·5°.

Expt. no.	Additives (μmoles)	O_2 consumed (μmoles)	Glucose consumed (μmoles)		Lactate formed (μmoles)		Aerobic inhibition (lactate) (%)
			Air	N_2	Air	N_2	
1*	—	0·40	—	—	0·03	0·16	81
	α-Ketoglutarate (10)	0·48	—	—	0·04	0·19	79
	α-Ketoglutarate (10), ATP, DPN	2·97	2·11	0·24	0·38	0·46	18
2†	ATP, DPN	0·98	—	—	0·42	0·85	51
	Pyruvate (4·5), L-malate (3·0) ATP, DPN	3·40	—	—	1·23	1·85	33
3‡	—	0·51	0·25	0·45	0·09	0·52	83
	ATP	1·34	0·96	0·43	0·11	0·72	85
	DPN	0·80	0·72	—	0·14	0·72	81
	ATP, DPN	3·57	1·55	0·55	0·47	0·96	51
4§	Pyruvate (7·5), L-malate (3)	0·40	—	—	0·18	1·16	84
	Pyruvate (7·5), L-malate (3), ATP	1·0	—	—	0·31	1·47	79
	Pyruvate (7·5), L-malate (3), DPN	0·85	—	—	0·20	1·20	83
	Pyruvate (7·5), L-malate (3), DPN, ATP	4·14	—	—	1·20	1·53	21

* Mitochondria: 150 mg-equivalent fresh tissue; glucose, 6 μmoles; incubation time, 35 min.
† Mitochondria: 200 mg-equivalent fresh tissue; glucose, 15 μmoles; incubation time, 30 min.
‡ Mitochondria: 200 mg-equivalent fresh tissue; glucose, 4·7 μmoles; incubation time, 40 min.
§ Mitochondria: 140 mg-equivalent fresh tissue; glucose, 15 μmoles; incubation time, 35 min.

of mitochondrial preparations. Respiration values were somewhat lower in the mitochondria prepared from the blend.

Thus, glycolytic activity was essentially the same in both types of mitochondrial preparations. In subsequent experiments, mitochondria prepared from blends were used. This preparation was also capable of carrying out oxidative phosphorylation (P : O ratio of 2·3, using α-ketoglutarate as substrate).

VOL. 5 1959/6

OL.
5
9/60

TABLE 4.—COMPARISON OF THE RESPIRATION AND OF THE GLYCOLYTIC ACTIVITY ASSOCIATED WITH MITOCHONDRIA PREPARED FROM POTTER–ELVEHJEM HOMOGENATES AND FROM NIRD BLENDS

Six brains (Expt. 1) and 4 brains (Expt. 2) were randomly halved; one half was homogenized in a NIRD blender, the other in a glass Potter–Elvehjem homogenizer fitted with a Perspex pestle (cf. text). Mitochondrial preparations were washed once and the 'fluffy layer' was kept. Incubation medium for glycolysis and respiration included 0·006 M-$MgSO_4$, 0·01 M-K-phosphate buffer, pH 7·4, 0·01 M-tris buffer, pH 7·4, 0·002 M-EDTA, 0·025 M-NAA, 0·005 M-glucose, 0·0066 M-α-ketoglutarate, 0·002 M-ATP and 0·00033 M-DPN. Total vol: 1·5 ml. Temperature: 37·5°. Enzymic activities expressed as μmoles/10 min/100 mg protein, as specified in Table 6.

Expt no.	Mitochondrial preparation	Respiration (μmoles O_2 consumed/hr/g fresh brain equivalent)	Glycolysis				Hexokinase	Aldolase	Lactic dehydrogenase (21°)
			(μmoles lactate formed/hr/g fresh brain equivalent)		Aerobic inhibition %	(μmoles glucose consumed/hr/g fresh brain equivalent) (aerobic)			
			Air	N_2					
1	Potter–Elvehjem	48	4·95	9·6	48	23	—	—	—
	NIRD blender	37	4·50	9·5	53	22	—	—	—
2	Potter–Elvehjem	—	—	—	—	—	72	490	75
	NIRD blender	—	—	—	—	—	80	530	73

Distribution of glycolytic enzymes in rat brain homogenates

The distribution of total glycolytic activity in brain homogenates, shown in Table 5, was based on a comparison of the values found for the mitochondria and for the supernatant fraction (cf. Experimental). About 10 per cent (7–13 per cent) of the glycolytic activity remained associated with the mitochondrial fraction. Similar figures were obtained when glycolytic activity of the mitochondrial fraction was compared with that of the total unfractionated homogenate (Table 5, Expt. 1, 5).

The mean percentage values obtained for the distribution of the individual glycolytic enzymes in the mitochondrial fractions were as follows: hexokinase, 34 (18–56); aldolase, 12 (7–27); TP-dehydrogenase, 9 (4–13) and lactic dehydrogenase, 11 (8–15). These results are summarized in Table 6.

The effect of washing on the glycolytic activity of brain mitochondria

It was found that 1–4 washings of the mitochondrial pellet with 0·025 M-sucrose solution (containing 0·025 M-NAA) did not remove glycolytic activity from the particles (Table 7). Moreover, these treatments did not modify the percentage aerobic inhibition of glycolysis.

Since some of the glycolytic enzymes are present in excess in the mitochondrial fraction, it was necessary to ascertain that the washing procedure did not simply remove excess enzymes, a possibility which can be overlooked when only the total glycolytic activity is measured.* It was found that washings did not decrease the specific activities of the glycolytic enzymes tested (hexokinase, aldolase, TP-dehydrogenase and lactic dehydrogenase) (Table 7). Thus, the glycolytic activity of the mitochondrial fraction reached a constant level after one washing and it was justified to subject the mitochondria to one thorough washing before use.

The effects of the 'fluffy layer' on some enzymic properties of brain mitochondria

A 'fluffy layer' ('light particles') was always found to remain loosely packed above the mitochondrial sediment ('heavy particles'). The 'fluffy layer' could be removed by gentle swirling; and by recentrifuging each fraction separately 2–3 times it was possible to obtain two fairly well-resolved particle fractions. (The 'light particle' fraction contained only about 10 per cent of the total protein content of the unfractionated mitochondria.)

Respiratory activity could not be detected in the 'light particle' fraction with the substrates used. The specific activities of aldolase and of lactic dehydrogenase were the same in the two particle fractions, but hexokinase activity was lower in the 'light particles.' The specific activities of total glycolysis, hexokinase and aldolase were essentially the same in the unfractionated and in the 'heavy particle' preparations. These results are summarized in Table 8. It is evident that removal of the 'fluffy layer' does not decrease glycolytic activity of brain mitochondrial preparations.

Attempts to dissociate glycolytic activity from brain mitochondrial preparations

Since glycolytic activity associated with 'sucrose mitochondria' could not be removed by several washings with 0·25 M-sucrose solution, it was of interest to test the effects of other media. The effects of treating mitochondria with Krebs–Ringer–phosphate medium on glycolysis were tested. Half of a batch of 'sucrose mitochondria'

* This suggestion was made by Professor F. Dickens.

VOL. 5 1959/6

BIOL. 5 6/60

TABLE 5.—RESPIRATION AND DISTRIBUTION OF GLYCOLYTIC ACTIVITY IN RAT BRAIN HOMOGENATE FRACTIONS

Mitochondria were prepared from Waring blends and washed once (Expt. 2 and 4), or twice (Expts. 1, 3, 5, 6). The 'fluffy layer' was always removed. HDP was added as a 'sparker' (1–1·5 μmoles/vessel), in Expt. 2 and 3 to supernatant fraction only, and in Expts. 1, 4, 5, 6 to all fractions. Glycolysis and respiration were assayed under the conditions specified in Table 4. For percent distribution calculation, supernatant + mitochondria = 100 per cent.

Expt. no.	Fraction	Respiration (μmoles O_2 consumed/hr/100 mg protein)	Glycolysis (μmoles lactate formed/hr/100 mg protein)		Aerobic inhibition %	Total anaerobic glycolytic activity/ fraction/hr	Distribution %
			Air	N_2			
1	Homogenate*	—	—	95	—	364	—
	Supernatant	—	238	238	0	289	93
	Mitochondria	110	24	40	40	21	7
2	Supernatant	—	198	204	3	260	87
	Mitochondria	87	11	35	69	38	13
3	Supernatant	—	—	246	—	373	92
	Mitochondria	185	—	101	—	31	8
4	Supernatant	—	—	243	—	434	90
	Mitochondria	—	—	65	—	46	10
5	Homogenate*	—	—	160	—	900	—
	Supernatant	—	—	284	—	582	93
	Mitochondria	182	44	72	39	41	7
6	Supernatant	—	—	342	—	900	89
	Mitochondria	—	83	131	37	112	11

* Homogenate = whole unfractionated suspension.

TABLE 6.—DISTRIBUTION OF GLYCOLYTIC ENZYMES AND OF TOTAL ACTIVITY IN BRAIN HOMOGENATE FRACTIONS

Mitochondrial preparations were washed 1–2 times and the 'fluffy layer' was removed in all but one experiment. Glycolysis was assayed as specified in Table 4, except that 'sparker' amounts of HDP (1–1·5 μmoles per vessel) were added when the supernatant was assayed. Temperature: 37·5°, except for lactic dehydrogenase which was measured between 18–21°. Enzymic activities expressed (per 10 min per 100 mg protein) as follows: hexokinase, μmoles glucose consumed; aldolase, μmoles triosephosphate produced; TP-dehydrogenase, μmoles lactate produced; lactic dehydrogenase, μmoles DPN reduced. No. of expts. given in brackets. % = per cent of total activity.

Fraction		Hexokinase		Aldolase		TP-dehydrogenase		Lactic dehydrogenase		Total glycolysis	
		Activity	%	Activity	%	Activity	%	Activity	%	Activity	%
Mitochondria	Mean	43(5)	34(5)	277(9)	12(7)	20(3)	9(3)	56(7)	11(7)	12·3(6)	9(6)
	Range	11–71	18–56	170–530	7–27	12–34	4–13	31–73	8–15	7–22	7–13
Supernatant	Mean	26(5)	66(5)	573(7)	88(7)	86(4)	91(3)	225(7)	88(7)	43·3(6)	90(6)
	Range	14–40	44–82	278–970	73–93	50–107	87–96	119–347	85–92	34–57	87–93

VOL. 5
1959/60

TABLE 7.—THE EFFECT OF SUCROSE WASHING ON THE ACTIVITY OF GLYCOLYTIC ENZYMES AND OF TOTAL GLYCOLYSIS IN BRAIN MITOCHONDRIA

Mitochondria were prepared from NIRD blends and the 'fluffy layer' was removed in Expts. 2 and 3. Respiration and glycolysis were measured as in Table 4. Enzyme assays were performed at 37·5°, except for lactic dehydrogenase which was measured at 18° (Expt. 2) and 19° (Expt. 3). Glycolytic and respiration activities are expressed as μmoles/100 mg protein/hr and the activities of individual enzymes, as μmoles/100 mg protein/10 min. % = per cent aerobic inhibition of lactate formation.

Expt. no.	Respiration				Glycolysis									Hexokinase				Aldolase				TP-dehydrogenase		Lactic dehydrogenase	
					1*			2*			3*														
	1*	2*	3*	4*	Air	N_2	%	Air	N_2	%	Air	N_2	%	1*	2*	3*	4*	1*	2*	3*	4*	1*	3*	1*	3*
1	217	190	200	—	20	37	46	16	46	64	27	46	42	—	—	—	—	—	—	—	—	—	—	—	—
2	108	—	135	—	12	33	63	—	—	—	14	34	59	70	—	75	—	226	—	189	—	5·6	6·1	53	70
3	—	—	—	—	11	35	68	—	—	—	15	37	60	—	—	—	—	222	—	213	—	—	—	64	80
4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	53	—	44	139	127	—	151	—	—	—	—

* No. of washings of mitochondria.

TABLE 8.—THE EFFECT OF THE 'LIGHT PARTICLE' FRACTION ON SOME ENZYMIC PROPERTIES OF BRAIN MITOCHONDRIA

The different preparations (cf. text) will be designated as follows: M = unfractionated mitochondria; H = heavy particles; L = light particles. Respiration and glycolysis measured as in Table 4. Enzyme assays (cf. Experimental) were performed at 37·5° except lactic dehydrogenase (18°). Glycolytic and respiratory activities expressed as μmoles/100 mg protein/hr and the activities of individual enzymes as μmoles/100 mg protein/10 min, as specified in Table 6; % = per cent aerobic inhibition of lactate formation.

Expt. no.	Respiration		Glycolysis						Hexokinase			Aldolase			Lactic dehydrogenase	
			M			H										
	M	H	Air	N_2	%	Air	N_2	%	M	H	L	M	H	L	H	L
1	164	148	13	33	60	19	39	51	—	—	—	—	—	—	—	—
2	74	67	9	29	69	18	36	50	30	31	—	173	217	—	—	—
3	—	—	—	—	—	—	—	—	42	45	—	139	145	—	—	—
4	—	—	—	—	—	—	—	—	—	70	11	—	246	220	—	—
5	—	—	—	—	—	—	—	—	—	—	—	—	226	235	62	61

VOL. 5 1959/60

BIOCHEM. J. (1960) 75, 9/60

TABLE 9.—THE EFFECTS OF SALINE TREATMENTS ON RESPIRATION AND GLYCOLYSIS OF BRAIN MITOCHONDRIA

All mitochondrial preparations derived from one batch of ten pooled brains as described in text. Experimental conditions as in Table 4. Glycolytic activity expressed as μmoles/100 mg protein/hr and activity of individual enzymes, as μmoles/100 mg protein/10 min, as specified in Table 6. % = per cent aerobic inhibition of lactate formation. Glycolysis, aldolase and TP-dehydrogenase activities measured at 37·5°, hexokinase at 37·5° (Expt. 1) and 30° (Expt. 2); lactic dehydrogenase, 20° (Expt. 1), 23° (Expt. 2).

Expt. no.	Mitochondrial preparation	Respiration (μmoles O_2/hr/100 mg protien	Glycolysis			Hexokinase	Lactic dehydro-genase	Aldolase	TP-dehydrogenase
			Air	N_2	%				
1	'Sucrose'	110	24	40	40	103	55		14
	'Sucrose-saline'	81	16	21	24	84	64		
	'Saline'	83	13	13	0	73	43		2·2
2	'Sucrose'	182	44	72	39	32*	47	303	25
	'Sucrose-saline'	78	21	33	27	27*	40	105	6
	'Saline'	100	25	28	11	24*	36	72	5

* In this experiment, hexokinase activity was assayed in a medium containing 0·02 M-K-phosphate buffer, pH 7·95, 0·008 M-ATP, 0·0046 M-$MgSO_4$, 0·002 M-glucose, 0·042 M-KCl and 0·01 M-NaF.

was subjected to two thorough washings with Krebs–Ringer–phosphate medium ('sucrose–saline' mitochondria), while the other half, twice washed with sucrose, served as control. 'Saline mitochondria' (cf. Experimental) were obtained from homogenates made in Krebs–Ringer–phosphate medium. Thus, three types of mitochondrial preparations were compared. All media contained 0·025 M-NAA and the three types of preparations were derived from one batch of ten pooled brains.

The results shown in Table 9 demonstrate that washing of 'sucrose mitochondria' with saline decreased glycolytic activity by about one-half, and at the same time resulted in a diminution of the aerobic inhibition of glycolysis. The decrease of glycolysis appears to be due to extraction of glycolytic enzymes from the mitochondria, since glycolytic activity was identical in the supernatant fractions of homogenates prepared in sucrose or saline solution (284 and 281 μmoles lactate/100 mg protein/hr, respectively). Treatment with saline caused little or no loss of hexokinase and lactic dehydrogenase activity, but extracted 75–85 per cent of TP-dehydrogenase and 65–76 per cent of aldolase activity. Nevertheless, aldolase activity remained in excess, and therefore the decrease in TP-dehydrogenase activity could not be due to the lack of lactic dehydrogenase or aldolase (cf. Experimental).

Preliminary attempts to dissociate glycolytic activity from brain mitochondria with 1% digitonin solution (COOPER and LEHNINGER, 1956) revealed that about 70 per cent of the original glycolytic activity could be extracted; however, the aerobic inhibition of glycolysis remained unaffected. (Control experiments have shown that digitonin does not inhibit glycolysis in brain supernatant.) A loss in respiratory activity of about 75 per cent was also noted using α-ketoglutarate as substrate.

DISCUSSION

The present results indicate that about 10 per cent of the glycolytic activity in rat brain homogenates is associated with the mitochondrial fraction (Table 5). This confirms the results of GALLAGHER, JUDAH and REES (1956) and of STRECKER (1957) demonstrating the ability of washed rat brain mitochondria to carry out glycolysis. HESSELBACH and DU BUY (1953) had previously reported similar observations and found that as much as 20 per cent of the glycolytic activity in mouse brain homogenates was associated with the mitochondrial fraction.

Other investigators have been unable to detect glycolytic activity in rat brain mitochondrial preparations (BRODY and BAIN, 1952; ALDRIDGE, 1957). Their data, however, do not make it sufficiently clear under what conditions their measurements of glycolytic activity were performed. In the present work, it was demonstrated that glycolytic activity in brain mitochondrial preparations depends on certain factors in the experimental conditions. Thus, aerobic conditions inhibited glycolysis, and this effect was more marked in the absence of either ATP or DPN (N.B. DPN was omitted in the assay system used by ALDRIDGE, 1957). In an assay system presumably containing cofactors but no added Krebs cycle intermediates (as used by BRODY and BAIN, 1952), aerobic lactate production is inhibited by about 50 per cent. From the results presented in Table 3, it is evident that optimal rates of glycolysis in brain mitochondria can be obtained only when the assay is performed anaerobically and in the presence of suitable concentrations of cofactors (0·00033 M-DPN, 0·002 M-ATP) and Krebs cycle intermediates (e.g. pyruvate).

VOL.
5
1959/6

Brain mitochondrial preparations obtained by differential centrifugation are not histologically homogeneous (BRODY and BAIN, 1952). Preliminary examinations of rat brain 'sucrose mitochondria' by interference microscopy* have revealed that, although this preparation consists predominantly of mitochondrial particles, some contamination by nuclei and by small cell-fragments is also detectable. Nevertheless, it was established that a part of the cell glycolytic activity is consistently spun down and associated with the fraction containing mitochondria.

It was found that several washings of the mitochondria with sucrose medium, as well as removal of the 'fluffy layer' ('light particles'), did not diminish glycolytic activity (Tables 7 and 8). Respiration could not be detected in the 'light particle' fraction with the substrates used.

Part of the glycolytic activity associated with brain mitochondria could be removed by washing with saline solution (Table 9). This loss was for the most part attributable to the removal of triosephosphate dehydrogenase (about 80 per cent of activity lost). Aldolase was also partly extracted by saline treatment, but its specific activity remained high in comparison with that of the triosephosphate dehydrogenase (ratio of specific activities, 15 : 1). On the other hand, hexokinase and lactic dehydrogenase were only slightly extracted by saline treatment (less than 20 per cent). Similarly, these two enzymes were not removed from the mitochondrial fraction by extraction with digitonin (unpublished data), although this treatment resulted in a 70 per cent loss of glycolytic activity. Thus it appears that lactic dehydrogenase and hexokinase associated with the mitochondrial fraction of brain are less susceptible to extraction than are the other glycolytic enzymes, even after disruption of the mitochondria (cf. digitonin fragments).

It was found that digitonin extraction reduced the oxygen consumption in the presence of α-ketoglutarate by about 75 per cent. It is known that digitonin causes the release of a number of Krebs cycle enzymes from mitochondria (COOPER and LEHNINGER, 1956). Washing the mitochondria with saline resulted in a 26–57 per cent decrease in respiration (substrate α-ketoglutarate). Since these treatments remove part of the enzyme systems of definite mitochondrial origin, it is not possible to decide whether the glycolytic enzymes which could be extracted were adsorbed on the mitochondrial particles or belonged to the mitochondrial structure.

With respect to the distribution of glycolytic enzymes, it is noted that the values obtained for aldolase and for triosephosphate dehydrogenase were similar to those reported by ABOOD *et al.* (1952). About 30 per cent of the homogenate hexokinase activity was found to be present in the mitochondrial fraction. This figure is much smaller than that reported by CRANE and SOLS (1953), but it is not clear whether the value of 87 per cent reported by these authors referred to the total high-speed residue or to the mitochondrial fraction alone.

Of particular interest was the finding that the specific activities of all the individual glycolytic enzymes tested except triosephosphate dehydrogenase were 9–40 times greater than that of the complete glycolytic system (Table 6). Triosephosphate dehydrogenase activity was of the same order of magnitude as glycolysis, suggesting a probable role of this enzyme as a pacemaker in the regulation of glycolysis as it occurs in brain mitochondrial preparations under the present experimental conditions. This observation is particularly relevant to the finding that this enzyme is the principal site of

* We acknowledge the help of Mr. G. B. David for performing these examinations.

the aerobic regulation of glycolysis in brain mitochondrial suspensions (BALÁZS and RICHTER, 1958; BALÁZS, 1959).

SUMMARY

1. 'Sucrose mitochondria' from rat brain are able to glycolyse glucose. Optimal conditions for the measurement of glycolysis are described.

2. The specific activities of the complete glycolytic system and of the individual glycolytic enzymes tested were similar in mitochondrial preparations obtained from blends or from Potter–Elvehjem homogenates; in the presence or absence of the 'light particle' fraction.

3. The distribution of glycolytic activity and of individual glycolytic enzymes (hexokinase, aldolase, TP-dehydrogenase, lactic dehydrogenase) in fractions of brain homogenates was measured.

4. About 10 per cent of the total glycolytic activity was associated with the mitochondrial fraction. The specific activity was not diminished by several washings with sucrose medium.

5. Extraction of mitochondria with Krebs–Ringer phosphate solution decreased glycolytic activity by about 50 per cent; this loss was mainly due to the removal of triosephosphate dehydrogenase. Digitonin extraction removed about 70 per cent of glycolytic activity. Neither type of extraction caused substantial extraction of hexokinase or lactic dehydrogenase from the mitochondrial preparations.

6. The specific activity of triosephosphate dehydrogenase was similar to that of the glycolytic system. The specific activities of the other enzymes tested were 9–40 times greater.

Acknowledgements.—The skilful technical assistance of Miss A. M. PHILLIPS and Mr. R. G. FISH is acknowledged. Thanks are due to Dr. D. RICHTER for his valuable suggestions and criticisms. This work was aided by a personal grant (R. B.) from the Rockefeller Foundation and a grant for equipment from the Medical Research Council.

REFERENCES

ABOOD L., GERARD R., BANKS J. and TSCHIRGI R. (1952) *Amer. J. Physiol.* **168,** 728.
ALDRIDGE W. N. (1957) *Biochem. J.* **67,** 423.
BALÁZS R. (1959) *Biochem. J.* **72,** 561.
BALÁZS R. and RICHTER D. (1958) *Biochem. J.* **68,** 5P.
BARKER S. B. and SUMMERSON W. H. (1941) *J. biol. Chem.* **138,** 535.
BRODY T. M. and BAIN J. A. (1952) *J. biol. Chem.* **195,** 685.
COOPER C. and LEHNINGER A. L. (1956) *J. biol. Chem.* **219,** 489.
CRANE R. K. and SOLS A. (1953) *J. biol. Chem.* **203,** 275.
DOUNCE A. L., BARNETT S. R. and BEYER G. T. (1950) *J. biol. Chem.* **185,** 769.
GALLAGHER C. H., JUDAH J. D. and REES K. R. (1956) *Biochem. J.* **62,** 436.
HESSELBACH M. and DU BUY H. (1953) *Proc. Soc. exp. Biol., N.Y.* **83,** 62.
LARDY H. A. (1955) *3ème Congrès International de Biochimie* p. 287. Academic Press, New York.
LEHNINGER A. L. (1951) In *Enzymes and Enzyme Systems* (Edited by Edsall J. T.) p. 1. Harvard University Press, Cambridge, Mass.
LONG C. (1952) *Biochem. J.* **50,** 407.
LOWRY O. H., ROSEBROUGH N. J., FARR A. L. and RANDALL R. J. (1951) *J. biol. Chem.* **193,** 265.
MEYERHOF O. and KIESSLING W. (1935) *Biochem. Z.* **283,** 83.
MEYERHOF O. and LOHMANN K. (1934) *Biochem. Z.* **271,** 89.
MEYERHOF O. and SCHULZ W. (1938) *Biochem. Z.* **297,** 60.
NEILANDS J. B. (1955) In *Methods in Enzymology* (Edited by COLOWICK S. P. and KAPLAN N. O.) Vol. 1, p. 449. Academic Press, New York.

VOL.
5
1959/6

NELSON N. (1944) *J. biol. Chem.* **153,** 375.
SIBLEY J. A. and LEHNINGER A. L. (1949) *J. biol. Chem.* **177,** 859.
SOLS A. and CRANE R. K. (1954) *J. biol. Chem.* **206,** 925.
SOMOGYI M. (1945) *J. biol. Chem.* **160,** 69.
STRECKER H. J. (1957) In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 469. Pergamon Press, London.
UMBREIT W. W., BURRIS R. H. and STAUFFER J. F. (1945) *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* Burgess, Minneapolis.

OL.
5
9/60

Journal of Neurochemistry, 1959, Vol. 5, pp. 18 to 31. Pergamon Press Ltd. Printed in Northern Ireland

QUANTITATIVE BIOCHEMICAL STUDIES OF WALLERIAN DEGENERATION IN THE PERIPHERAL AND CENTRAL NERVOUS SYSTEMS—I

CHEMICAL CONSTITUENTS*

RICHARD E. McCAMAN,†‡ and ELI ROBINS
Departments of Pharmacology and of Psychiatry and Neurology, and the Beaumont-May Institute of Neurology, Washington University School of Medicine, St. Louis, Missouri

(*Received* 26 *February* 1959)

VOL.
5
1959/6

SINCE the pioneer studies of NOLL (1899) and MOTT and HALLIBURTON (1901) on the chemical changes in degenerating nerve, many refinements in the methods of fractionating and determining the chemical constituents of nervous tissue have been made. BRANTE (1949) was one of the first to study the changes of various lipid fractions in degenerating peripheral nerve using methods which determine the lipids as they are known today. These results have been greatly extended by ROSSITER and co-workers who have studied, in addition to the changes in the lipids, the changes in various phosphorus fractions and enzymic activities in degenerating sciatic nerve of the cat (JOHNSON, MCNABB and ROSSITER, 1949; LOGAN, MANNELL and ROSSITER, 1952*b*; HOLLINGER, ROSSITER and UPMALIS, 1952; HOLLINGER and ROSSITER, 1952). However, there has apparently been no report on the quantitative chemical changes during Wallerian degeneration in a central white tract.

The study of chemical changes in degenerating central tracts as well as in peripheral nerve has been greatly facilitated in the present study by utilizing recent modifications and extensions of the original Linderstrøm-Lang and Holter quantitative histochemical method (LOWRY, 1953; ROBINS, 1957). The advantages of this method, as compared with the classical technique of gross dissection and homogenization of the nerve tissue, include: (a) the use of highly sensitive microchemical methods which permits the chemical analysis of a great many different chemical constituents of small central tracts or of a single peripheral nerve trunk; (b) chemical constituents and enzymes are stable in frozen-dried material for years; thus one can return to the same material repeatedly; (c) close visual control of dissection eliminates the variability introduced by epineural connective tissue, meninges, major blood vessels, and adjacent nervous tissue. Unless the quantitative histochemical method was used, serious technical difficulties would arise both in getting enough control tissue for analysis and in dissecting an anatomically defined central tract.

In this report, the changes in the concentrations of total lipids, 6 lipid fractions, phosphorus and amino-nitrogen fractions, and protein of a peripheral nerve (tibial

* Supported in part by a grant from the National Multiple Sclerosis Society.

† The data presented in this paper formed part of a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Washington University, 1957.

‡ Present address: The Institute of Psychiatric Research, Indiana University Medical Center, Indianapolis, Indiana.

nerve) as well as a central white tract (optic nerve) undergoing Wallerian degeneration are presented. A preliminary report of some of these results has appeared (McCaman and Robins, 1958). In the second paper of this series, the changes in the enzymic activities of 12 enzymes during Wallerian degeneration are reported (McCaman and Robins, 1959). A related study on the morphological changes in these degenerating nerves as observed with the electron microscope will be reported elsewhere (Luse and McCaman, in press).

MATERIALS AND METHODS

Adult albino rabbits were used for this study. A total of 20 animals were subjected to an enucleation of the left eye and transection of the tibial nerve on the left side. All surgical operations were performed under aseptic conditions. The eye was removed with a minimum of trauma to the optic nerve by making a circular incision along the corneal limbus, separating the conjunctiva from the bulb, cutting the tendons of the recti and oblique muscles, rotating the bulb slightly down and forward, and cutting the optic nerve with a pair of curved enucleation scissors. Bleeding was slight when the transection of the nerve was made proximal to the venous sinus which is located at the point of entry of the nerve into the globe. The sciatic nerve was exposed by incising the skin and fascia on the lateral surface of the leg and separating the biceps and quadriceps muscles by blunt dissection. The tibial–sural complex was separated from the peroneal nerve and ligated just below the level of the greater trochanter. After the nerves were cut distal to the ligation, the central ends were drawn into a pocket made in the overlying muscle and sutured to minimize the possibility of regeneration. By sparing the peroneal nerve the complications of trophic foot sore and a serious state of debilitation, which occur after section of the whole sciatic nerve, were largely avoided.

After periods of 14, 45, 100 and 200 days, the animals were sacrificed by decapitation and the optic and tibial nerves were removed. The first 5 mm of the tibial nerve distal to the cut were discarded to avoid tissue subjected to the trauma of the original operation. Only the intracranial portion of the optic nerve, extending from the optic foramen to the chiasma, was used in this study. The pieces of fresh nerve, weighing approximately 5 mg (optic) and 15 mg (tibial), were obtained as quickly as possible after death of the animal and frozen in liquid nitrogen. The nerves were allowed to warm to $-20°$ in a cold room, mounted and cut into sections 25 μ and 50 μ thick, as described by Lowry (1953), without the temperature ever being allowed to rise above $-15°$. Once the sections were cut, they were dehydrated under vacuum (0·001 mm) overnight at a temperature not exceeding $-35°$. After dehydration, the sections could be stored for months or years at $-25°$ without deterioration.

The dissection of the frozen-dried tissue was carried out at room temperature under a dissecting microscope. The optic nerves, freed of meninges and major blood vessels, and the tibial nerves, freed of all epineural connective tissue, were considered to be homogeneous, and samples were taken at random within a given section. The pieces of tissue analysed varied from 0·7 μg to 50 μg, dry weight, depending on the constituent measured and the time of degeneration. Methods for determining dry weight and volume have been described (Lowry, 1953).

The standard errors of the mean listed in Tables 1–5 are derived from multiplicate determinations from several nerves. Thus, they do not give an index of either methodological variability or biological variability, but rather a composite of both. A *P*-value of 0·01 or less was accepted as being statistically significant.

Chemical methods. Methods for measuring lipid fractions (Robins, Lowry, Eydt and McCaman, 1956), phosphorus fractions (Lowry, Roberts, Leiner, Wu and Farr, 1954), protein (Lowry, Rosebrough, Farr and Randall, 1951), cholesterol (Albers and Lowry, 1955), and deoxyribonucleic acid (DNA) (Kissane and Robins, 1958) have been given.

Total nucleic acid in the optic nerve was determined by a micro modification of the Logan, Mannell and Rossiter (1952*a*) ultraviolet absorption procedure. After precipitating and washing the brain tissue (2 to 10 μg) with cold 0·3 N-trichloroacetic acid (TCA) and extracting the lipids, the residue was heated at 90° in 5 μl of 0·3 N-TCA for 15 min. A 4 μl aliquot was diluted to 50 μl with water and the absorbance at 268·5 mμ was measured in the Beckman spectrophotometer. On other pieces of tissue from the same nerves, phosphorus, instead of ultraviolet absorbance, was measured on the supernatant fluid after the hot TCA treatment. As will be shown and discussed later the phosphorus values do not agree with the ultraviolet values.

Acid-soluble free amino-nitrogen was determined by a procedure similar to that used for the cephalin fraction of the lipid procedure (ROBINS, LOWRY, EYDT and MCCAMAN, 1956). The supernatant fluid (10 μl), removed after precipitating the pieces of tissue (1–10 μg) with 0·3 N-TCA at 4°, is allowed to react with 3:5-dinitrofluorobenzene (DNFB) at pH 9·1 for 30 min at 60°. After dilution with 2 N-HCl to a final volume of 50 μl, the solution is read in a Beckman spectrophotometer at 420 mμ. Serine is used as a working standard; it has a molar extinction coefficient of 4500 under these conditions. Each individual amino acid gives a somewhat different extinction coefficient in this method, but the value of 4500 obtained for serine is about the mean.

Although DNFB will react with compounds other than those with free amino groups, the concentration of these compounds appears to be quite small compared to the concentration of free amino-nitrogen in the acid wash. LOWRY (personal communication) has observed that more than 95 per cent of the acid-soluble DNFB-reacting material of rabbit brain homogenates can be accounted for as free amino acids.

RESULTS

Comparison of control tibial and optic nerves

The tibial nerve contains approximately 20 per cent more (total) lipid and less protein per unit dry weight than does the optic nerve (Table 1, column A). Among the lipid fractions, cephalins, P-free sphingolipids and cholesterol show approximately this same ratio in the 2 nerves (Table 2, column A). Accordingly, the mole fractions of these 3 lipid fractions are not appreciably different in the 2 nerves: cephalins (tibial 0·27; optic 0·24), P-free sphingolipids (0·12; 0·13), and cholesterol (0·41; 0·44). The mole fraction of each lipid fraction has been defined as

$$\frac{\text{m-moles of each lipid fraction}}{\text{m-moles total lipid (cholesterol + total lipid P + P-free sphingolipids)}}$$

In contrast to these 3 fractions, lecithins and sphingomyelins show appreciable differences between the 2 nerves (Table 2, column A). Sphingomyelin is 159 per cent greater in the tibial than in the optic nerve (mole fractions, 0·16 and 0·08, respectively). Lecithins show a reversal of the relative abundance, being 22 per cent *less* abundant in the tibial than in the optic nerve (mole fractions, 0·07 and 0·11, respectively).

Both inorganic + labile and organic acid-soluble phosphorus are approximately 50 per cent greater in the optic nerve than in the tibial nerve (Table 3, column A). This difference probably reflects the generally greater enzymic activities (MCCAMAN and ROBINS, 1959) and higher metabolic turnover in the optic nerve. The approximately 50 per cent higher acid-soluble amino-N (almost exclusively amino acids) in the optic nerve (Table 3, column A) may also reflect this difference in metabolic levels or may indicate certain differences in amino acid and protein metabolism between central and peripheral white matter.

The optic nerve has approximately 50 per cent more cells than does the tibial nerve (Table 4, column A), whether the reference is fat-free dry weight or volume (Table 1, column A).

Comparison of degenerating tibial and optic nerves

The chemical fractions were determined directly as a function of dry weight. However, the lipids amount to more than 50 per cent of dry weight, and their subsequent loss results in a marked change in the dry weight of the nerves during degeneration. Therefore, the results are recorded in terms of the fat-free dry weight at each period of degeneration. The chemical changes during degeneration could

VOL. 5 1959/6

have been expressed with volume as a reference without appreciably changing the results (Table 1). Volume was not used because oedema is known to occur in degenerating nerves (JOHNSON, MCNABB and ROSSITER, 1949) and because any distortion during the removal and freezing of the nerve or small variations in the thickness of the microtome sections might increase the variability of the results.

TABLE 1.—FAT-FREE DRY WEIGHT, PROTEIN, AND TOTAL DRY WEIGHT PER UNIT VOLUME OF CONTROL AND DEGENERATING TIBIAL AND OPTIC NERVES

All values are expressed as g/kg total dry wt ± standard error of the mean, unless otherwise indicated. Each mean value is the result of 3–6 determinations on tissue from each of 6 animals for the control and 2–4 animals for each period of degeneration.

Nerve	Constituent	Control A	Days after section			
			14	45	100	200
Tibial	Fat-free dry wt	310 ± 10	430 ± 10*	540 ± 30*	670 ± 20*	
	Protein	347 ± 6	445 ± 12*	544 ± 19*	544 ± 24*	
	Dry wt/vol (g/l.)	401 ± 9	267 ± 6*	217 ± 16*	171 ± 4*	
	Fat-free dry wt/l.†	124	115	117	115	
Optic	Fat-free dry wt	390 ± 7	400 ± 5	380 ± 8	500 ± 20*	600 ± 30*
	Protein	437 ± 11	437 ± 13	385 ± 8*	550 ± 19*	651 ± 8*
	Dry wt/vol (g/l.)	365 ± 6	420 ± 6*	388 ± 13	297 ± 18*	286 ± 11*
	Fat-free dry wt/l.†	142	168	147	148	172

* Significantly different ($P < 0{\cdot}01$) when compared with control value. In the optic nerve, the significant decrease in protein at 45 days and the significant increase in dry wt/l. at 14 days are inconsistent with the remainder of the results and are unexplained.

† Calculated by $\frac{\text{Row 1} \times \text{Row 3}}{1000}$ for each nerve separately.

Fat-free dry weight (Table 1). A marked difference between the proportion of fat-free dry weight to total dry weight of degenerating tibial and optic nerves is apparent. The relative fat-free dry weight of the tibial nerve increases markedly over the control value as early as 14 days and continues to increase until at least 100 days after section of the nerve. There is, in contrast, no statistically significant change in the degenerating optic nerve at 14 or 45 days when compared to the control value. There is a definite increases at 100 days and a further increase at 200 days. This represents, however, only a 54 per cent increase in fat-free dry weight in the 200-day degenerating optic nerve compared to a 116 per cent increase in the 100-day tibial nerve (Table 6). The increase in protein parallels the change in fat-free dry weight with the exception of the value for 100-day tibial nerve. This latter discrepancy may be attributed to the presence of an appreciable amount of collagen at this time (ABERCROMBIE and JOHNSON, 1946*a*), since collagen gives less colour with the protein reagent than many other proteins (LOWRY *et al.*, 1951).

Lipid fractions (Table 2). The loss of myelin is one of the most prominent characteristics of Wallerian degeneration. Since myelin is composed in large part of lipids, the measurement of various lipid fractions serves to indicate the extent of degeneration. In the tibial nerve, each fraction, except cholesterol, has decreased by 50 per cent or more at 14 days (Table 6). All lipid fractions of the tibial nerve continue to show a decrease until at least 100 days. At this time, the lipids have

TABLE 2.—LIPIDS OF DEGENERATING TIBIAL AND OPTIC NERVES

All values are expressed as m-moles of lipid/kg fat-free dry wt ± standard error of the mean. The means represent 4–7 determinations on each of 6 nerves for the control and 2–4 nerves for each of the various times after section of the nerve.

Nerve	Constituent	Control A	Days after section			
			14	45	100	200
Tibial	Cephalins	768 ± 21	286 ± 7*	100 ± 7*	40 ± 3*	
	Lecithins	200 ± 3	95 ± 7*	78 ± 6*	34 ± 3*	
	Sphingomyelins	458 ± 12	188 ± 5*	92 ± 4*	25 ± 4*	
	P-free sphingolipids†	361 ± 18	181 ± 7*	91 ± 9*	12 ± 1*	
	Total P-lipid	1390 ± 21	575 ± 12*	270 ± 6*	101 ± 6*	
	Cholesterol	1190 ± 30	862 ± 23*	425 ± 13*	122 ± 21*	
Optic	Cephalins	550 ± 10	488 ± 12*	447 ± 22*	240 ± 22*	184 ± 12*
	Lethicins	256 ± 5	225 ± 12	276 ± 8	170 ± 8*	118 ± 13*
	Sphingomyelins	177 ± 5	190 ± 5	200 ± 8	90 ± 6*	64 ± 3*
	P-free sphingolipids†	300 ± 5	298 ± 22	310 ± 20	188 ± 14*	52 ± 10*
	Total P-lipid	967 ± 12	900 ± 20*	945 ± 32	510 ± 24*	367 ± 13*
	Cholesterol	985 ± 15	930 ± 22	1200 ± 22*	738 ± 30*	310 ± 35*

* Significantly different ($P < 0.01$) when compared with control values. The significant increase in cholesterol in optic nerve at 45 days is inconsistent and unexplained.

† Probably chiefly cerebrosides.

decreased to from 3 to 17 per cent of the control values (Table 6). Relative to the changes of the sphingomyelin and P-free sphingolipid fractions which show essentially the same percentage changes, cholesterol undergoes a less rapid decrease initially; lecithins show a less rapid decrease after day-14 and remain at the highest level (17 per cent) at 100 days; and cephalins decrease more abruptly than the other fractions (Table 6).

The changes in the degenerating optic nerve contrast strikingly with those in the tibial nerve. In the optic nerve there is a negligible loss of lipid until 100 days. Although the decreases in cephalins at 14 and 45 days are statistically significant when compared with the control values, these decreases are slight when compared with the marked decreases found in tibial nerve at these times. Even at 200 days the optic nerve still contains from 17 to 46 per cent of the original amount of lipid (Table 6). This is 3 to 5 times more lipid than was found in the 100-day tibial nerve. The changes of the cholesterol, lecithins, and cephalins relative to the sphingomyelins and P-free sphingolipids were similar to those described for the tibial nerve.

VOL. 5 1959/6

Acid-soluble P *fractions and amino*-N (Table 3). The changes in the acid-soluble phosphorus and amino-nitrogen fractions during degeneration were less marked than in the lipid fractions. In the tibial nerve the two acid-soluble P fractions and amino-N fraction (chiefly free amino acids) showed increases of from 22 to 60 per cent at 14 days and had decreased at 100 days to values not much different from the control values. Recently PORCELLATI and THOMPSON (1957) have reported similar changes in the level of free amino acids after section of the sciatic nerve in the hen. In the optic nerve these constituents reached a maximum later, at 45 or 100 days, and showed a maximal increase of from 20 to 40 per cent (Table 6).

TABLE 3.—ACID-SOLUBLE FRACTIONS OF DEGENERATING TIBIAL AND OPTIC NERVES

All values are expressed as m-moles of phosphorus or amino-nitrogen/kg fat-free dry wt ± standard error of the mean. The means represent 5 determinations on each of 6 nerves for the control and 2–4 nerves for each of the various times after section of the nerve.

Nerve	Fraction	Control A	Days after section			
			14	45	100	200
Tibial	Acid-soluble P					
	Inorganic + labile	48 ± 3	60 ± 5	48 ± 4	42 ± 1	
	Organic	87 ± 3	118 ± 5*	102 ± 6	92 ± 4	
	Acid-soluble amino-N	181 ± 6	290 ± 14*	242 ± 11*	225 ± 3*	
Optic	Acid-soluble P					
	Inorganic + labile	72 ± 2	72 ± 2	71 ± 2	100 ± 8*	80 ± 7
	Organic	126 ± 2	145 ± 2*	176 ± 2*	158 ± 6*	152 ± 7*
	Acid-soluble amino-N	282 ± 8	305 ± 8	326 ± 15	338 ± 18*	337 ±27

* Significantly different ($P < 0{\cdot}01$) when compared with control value.

Numbers of cells (*DNA*). The changes in DNA concentration (number of cells) in the two nerves show that the process of cellular invasion and multiplication is more rapid in the tibial than in the optic nerve (Table 4). The greater rapidity of cellular invasion in the tibial nerve is similar in time to the more rapid loss of lipid described above in the tibial nerve. In the tibial nerve the concentration of DNA increases markedly by 14 days to a value which is 271 per cent higher than that of the control. This maximal value corresponds to 260×10^{10} cells per kg fat-free dry weight. There is a decrease in the concentration of DNA from 14 to 45 days and there is no significant difference between the 45- and 100-day values. These changes in the concentration of DNA in the degenerating tibial nerve are similar to those reported by LOGAN *et al.* (1952*b*) for degenerating cat sciatic nerve.

The pattern of change in DNA in the degenerating optic nerve is different from that in tibial nerve. At 14 days, DNA has increased only 33 per cent. There is similar increase from 14 to 45 days, but only a slight increase, 19 per cent, which is not statistically significant, from 45 to 100 days. From 100 to 200 days there is an

additional 62 per cent increase (Table 6). The maximal value for DNA of the optic nerve is seen at 200 days, in contrast to 14 days for the tibial nerve. The absolute increase in DNA is only 73 per cent of that found for the tibial nerve. It is of considerable interest, however, that (because there are more cells to begin with in the optic nerve) the maximal number of cells is essentially the same for the two nerves (260×10^{10} *vs.* 255×10^{10}).

TABLE 4.—NUCLEIC ACIDS AND RESIDUAL PHOSPHORUS OF DEGENERATING TIBIAL AND OPTIC NERVES

All values are expressed as m-moles of P/kg fat-free dry wt ± standard error of the mean. Each mean value is the result of 3–6 determinations on each nerve of 6–8 animals for the control and 2–4 animals for the degenerating nerves, unless otherwise indicated.

Nerve	Constituent	Control A	Days of degeneration			
			14	45	100	200
Tibial	DNA phosphorus**	14 ± 1	52 ± 2*	32 ± 2*	28 ± 1*	
	No. of cells × 10^{-10}/kg***†	70	260	160	140	
	Residual phosphorus	150 ± 4	75 ± 4*	45 ± 4*	38 ± 2*	
Optic	DNA phosphorus**	21 ± 1	28 ± 1*	34 ± 4*	38 ± 1*	51 ± 1*
	No. of cells × 10^{-10}/kg***†	105	140	170	190	255
	Nucleic acid P****†					
	a. UV absorption*****†	52 ± 2	74 ± 1	83 ± 3	93 ± 3	
	b. P determination†	83 ± 2	103 ± 4	116 ± 14	98 ± 4	
	RNA (UV-DNA)†	31	46	49	55	
	Residual P	110 ± 4	105 ± 7	92 ± 3*	54 ± 2*	42 ± 2*

* Significantly different ($P < 0.01$) when compared with control values.

** Calculated from separate fluorometric determinations, it being assumed that DNA contains 9 per cent phosphorus.

*** Calculated from DNA values with a value of 7×10^{-12} gm of DNA/cell. (HELLER and ELLIOT, 1954). This is equivalent to 2×10^{-14} moles P (DNA)/cell.

**** Each value for total nuclei acid P is the mean of 5 determinations on each nerve of 3 animals or the control and for 1 animal for each period of degeneration.

***** A molar extinction coefficient of 10,000 at 268·5 mμ for nucleic acid phosphorus was used.

† Statistical calculations not made.

Total nucleic acid and RNA. Total nucleic acid in the optic nerve was measured by the ultraviolet absorption procedure of LOGAN, MANNELL and ROSSITER (1952*a*). These investigators have shown that the procedure of SCHNEIDER (1945) yields falsely high values for total nucleic acid phosphorus in tibial nerve of the cat, whereas the ultraviolet procedure gives lower, and presumably correct values. In the present study in optic nerve similar results were obtained on the control nerves and at all times of degeneration, except at 100 days where the 2 methods of measurement were in agreement. At all other times the total nucleic acid phosphorus values were higher by the Schneider procedure than those obtained by ultraviolet absorption (Table 4). Since the fluorometric method of KISSANE and ROBINS (1958) for DNA and the ultraviolet absorption method for total nucleic acids (LOGAN, MANNELL and ROSSITER, 1952*a*) have been shown to be valid procedures, the difference between the two values

should give an accurate estimate of RNA for degenerating optic nerve. The increase in RNA content of degenerating optic nerve is essentially the same as that in DNA, and as a result the RNA to DNA ratio is maintained at the relatively constant value of 1·5, at least to 100 days of degeneration (Table 4).

Phosphoinositides and phosphoprotein. Previous reports by LOGAN, MANNELL and ROSSITER (1952*a*), LEBARON and FOLCH (1956), and HUTCHISON *et al.* (1956) have shown the presence of phosphoinositides in the central nervous system. LOGAN *et al.* (1952*a*) have shown that the procedure of SCHNEIDER (1945) for the fractionation of

TABLE 5.—'PHOSPHOINOSITIDE,' NUCLEIC ACID, AND 'PHOSPHOPROTEIN' FRACTIONS OF DEGENERATING OPTIC NERVES

All values are expressed as m-moles of phosphorus/kg fat-free dry wt ± standard error of the mean. Each value represents the mean of 4–5 determinations on each nerve of 2–4 animals.

Fraction	Control	Days of degeneration			
		14	45	100	200
'Phosphoinositides'†	121 ± 6	112 ± 3	102 ± 9	54 ± 1*	36 ± 2*
Total nucleic acid (determined as P)	27 ± 1	46 ± 1*	46 ± 3*	65 ± 4*	72 ± 3*
'Phosphoprotein'	30 ± 2	26 ± 2	19 ± 2*	27 ± 2	36 ± 2

* Significantly different ($P < 0{\cdot}01$) when compared with control values.
† Phosphorus extracted by chloroform–methanol–HCl after acid-soluble and lipid phosphorus fraction have been removed.

tissue phosphorus yields erroneously high values for total nucleic acid phosphorus and phosphoprotein phosphorus. They indicate that the error in the determination of these two fractions is the result of a partial hydrolysis of the phosphoinositides by hot acid. They propose the use of ultraviolet absorption rather than phosphorus determination for total nucleic acids and thus avoid the erroneous nucleic acid values.

The incomplete hydrolysis of the phosphoinositides deprives the phosphorus values for the nucleic acid and residual phosphorus fractions (Table 4) of any quantitative meaning. However, the decrease in residual phosphorus of degenerating tibial and optic nerve and the agreement between the 100-day values in optic nerve for total nucleic acid as measured by ultraviolet absorption and phosphorus determination strongly suggest that the phosphoinositides decrease with degeneration. This has previously been shown to occur in degenerating peripheral nerve by LOGAN *et al.* (1952*b*).

To obtain a more reliable measure of phosphoinositides and study their changes in degenerating optic nerve, a separate group of samples were subjected to the following procedure. The samples were washed with cold TCA and treated with ethanol to remove the acid-soluble and lipid phosphorus, respectively. The remaining residue was treated twice at room temperature with 20 μl of chloroform–methanol–hydrochloric acid (200 : 100 : 1 by volume). This solvent system has been shown to completely extract the phosphoinositides (LEBARON and FOLCH, 1956). The two chloroform–methanol–hydrochloric acid fractions were combined and evaporated, and the total phosphorus in this fraction is reported as phosphoinositides (Table 5).

The residue left after the chloroform–methanol–hydrochloric acid treatment was subjected to hot TCA hydrolysis for 15 min at 90°. The soluble phosphorus appearing in the supernatant fluid was assumed to be total nucleic acid phosphorus. The residue still remaining was washed twice with cold 0·3 N-TCA to remove contaminating soluble nucleic acid phosphorus and ashed directly to give residual phosphorus (Table 5).

The phosphoinositide fraction of degenerating optic nerve decreased only 16 per cent during the first 45 days. From 45 to 100 days there was a more marked decrease of 40 per cent and a further decrease of 15 per cent from 100 to 200 days. This fraction, therefore, decreases during optic degeneration in a manner which is strikingly similar to the decrease observed for the other lipid fractions of optic nerve.

The phosphorus of the nucleic acid fraction, obtained subsequent to the chloroform solvent extraction, continues to increase at least until 200 days of degeneration (Table 5). The 200-day value is 167 per cent higher than the control value. This trend is consistent with that of the ultraviolet determinations for total nucleic acids reported in Table 4. The lower absolute values for total nucleic acid reported in Table 5 suggest that chloroform–methanol–hydrochloric acid extracts some of the nucleic acid phosphorus. For this reason the values listed for phosphoinositides may be too large by about 30 m-moles per kg dry weight.

After removal of the phosphoinositides and the nucleic acids, the residual phosphorus (Table 5) probably consists entirely of the classically defined phosphoproteins (SCHMIDT and THANNHAUSER, 1945; LOGAN, MANNELL and ROSSITER, 1952*a*). With the exception of an apparent decrease at 45 days, there is no appreciable change in the concentration of this fraction during degeneration of the optic nerve. These results are the same as those observed for a similar fraction in degenerating peripheral nerve (LOGAN, MANNELL and ROSSITER, 1952*b*).

DISCUSSION

Comparison of control tibial and optic nerves. The finding that the optic nerve contains less total lipid per unit dry weight than the tibial nerve cannot be generalized to the entire central nervous system. MCDOUGAL (1958) has recently shown that there are significant differences among various central tracts with regard to total lipid content. The lipid content of optic nerve as found in this study is similar to that found by MCDOUGAL in the mammillothalamic tract and the small-fibre portion of the optic tract. The greater lipid content in the tibial nerve, however, is similar to that in the dorsal columns, the large-fibre portion of the optic tract, and the olfactory tract (MCDOUGAL, 1958).

On the other hand, the finding of the relatively low mole fraction of sphingomyelin in the optic nerve as compared with the tibial nerve appears to be a finding which is generally true throughout the central and peripheral nervous systems. The lower mole fraction of sphingomyelin in the white matter of the central nervous system has been found in 7 sites in 4 species: the subjacent white matter of the monkey cerebellum (ROBINS, EYDT and SMITH, 1956), the subjacent white matter of the motor and visual areas of the monkey cerebral cortex (ROBINS, SMITH and EYDT, 1956), the cerebral white matter in man and in the cow (BRANTE, 1949), alveus of Ammon's horn in the rabbit (LOWRY, ROBERTS, LEINER, WU, FARR and ALBERS, 1954), the optic nerve of the cow (BRANTE, 1949), and the white matter of the spinal cord of the rabbit and

VOL.
5
1959/6

TABLE 6.—FAT-FREE DRY WT, LIPID FRACTIONS, NUMBERS OF CELLS, PHOSPHORUS FRACTIONS, AND AMINO-NITROGEN VALUES IN DEGENERATING TIBIAL AND OPTIC NERVES

All values are expressed as a percentage of the control value (0-day) for each nerve, which was taken as 100 per cent. These percentages were calculated from the data in Tables 1–4.

Constituent	Nerve Tibial (T) Optic (O)	Days of degeneration				
		0	14	45	100	200
Fat-free dry wt	T	100	139	174	216	
	O	100	103	98	128	154
Dry wt/l.	T	100	67	54	43	
	O	100	115	107	82	79
Fat-free dry wt/l.	T	100	93	94	93	
	O	100	118	104	104	121
Lipid fractions						
Cephalins	T	100	37	13	5	
	O	100	89	81	44	34
Lecithins	T	100	47	39	17	
	O	100	88	108	67	46
Sphingomyelins	T	100	41	20	5	
	O	100	107	113	51	36
P-free sphingolipids	T	100	50	25	3	
	O	100	100	104	63	17
Total lipid P	T	100	44	19	17	
	O	100	93	98	53	38
Cholesterol	T	100	72	36	10	
	O	100	94	122	75	32
Number of cells (DNA)	T	100	371	229	200	
	O	100	133	162	181	243
P fractions						
Inorganic + labile P	T	100	122	100	86	
	O	100	100	99	139	111
Organic acid-soluble P	T	100	136	117	105	
	O	100	115	140	125	121
Acid-soluble NH_2—N	T	100	160	134	125	
	O	100	108	116	120	120

cow (BRANTE, 1949). The higher mole fraction of sphingomyelin in peripheral nerve has been found in 3 sites in 3 species: the sciatic nerve of the cat (JOHNSON, MCNABB and ROSSITER, 1949) and of the cow and rabbit (BRANTE, 1949), and the ventral root (intradural) and dorsal root (intradural) of the cow (BRANTE, 1949).

The only reported equivocal result for any central tract or peripheral nerve is BRANTE'S (1949) for the vagus of the cow. The mole fraction of sphingomyelin, without his 'unidentified lipid fraction', is 0·10, which is still higher than that of any central white matter but lower than that of any other peripheral nerve. It may be that largely unmyelinated peripheral nerves are somewhat different, but more data are needed to settle this point.

The presumably greater metabolic potential of the optic nerve compared with the tibial nerve, as indicated by the higher levels of inorganic + labile and organic acid-soluble phosphorus and of amino-N in the optic nerve, is a finding which may be a general property of central tracts as compared with peripheral nerves. LOWRY, ROBERTS, LEINER, WU, FARR and ALBERS (1954) have found high levels of acid-soluble phosphorus fractions in alveus, comparable to those found in the optic nerve in this study. Conversely, in this laboratory (unpublished) we have found low levels of inorganic + labile and organic acid-soluble phosphorus and of amino-N in the abdominal vagus nerve of the rabbit—37, 91 and 148 m-moles per kg fat-free dry weight, respectively. Since the abdominal vagus in the rabbit is almost completely unmyelinated, it provides a kind of peripheral nerve, completely different from the tibial, for study. In spite of these histological differences the vagus and tibial nerves are very similar with regard to these fractions and very different from optic nerve and alveus.

Degeneration in the peripheral nervous system. Previous chemical investigations of degenerating sciatic nerves of the rabbit (BRANTE. 1949), cat (JOHNSON, MCNABB and ROSSITER, 1949), and the rat (MANNELL, 1952) have demonstrated a marked decrease in the concentration of the individual lipid fractions. The present study of a single branch of the sciatic trunk (tibial nerve) has yielded essentially the same results as the previous studies. There is a marked and early reduction of all lipid fractions, with the most dramatic changes occurring during the first 14 days. The reduction in residual phosphorus and small increase in acid-soluble phosphorus observed in the present study are similar to the changes reported by LOGAN, MANNELL and ROSSITER (1952*b*) for cat sciatic nerve. The present study indicates that the greater part of the increase in acid-soluble phosphorus is due to an increase in the organic fraction.

The increase in DNA phosphorus observed in the degenerating tibial nerve in the present study is similar to that reported by LOGAN *et al.* (1952*b*). The use of DNA as an index of the number of cell nuclei (Table 4) provides an interesting comparison with previous histological studies. When the number of cells per mm^3 in the 14-day tibial nerve is calculated,* a value of $3{\cdot}0 \times 10^5$ is obtained. This closely agrees with the quantitative histological results summarized by JOSEPH (1950). Quantitative histologic counts on 5 different peripheral nerves undergoing Wallerian degeneration have indicated that the maximal cell density is essentially the same for all of them, i.e. $3{\cdot}3$–$4{\cdot}7 \times 10^5$ cells per mm^3.

* 430 g fat-free dry wt/kg dry wt × 267 g dry weight/l. = 115 g fat-free dry wt/l.; $260 \times 10^{10} \times 0{\cdot}115 \times 10^{-6} = 3{\cdot}0 \times 10^5$ cells/mm^3.

VOL. 5 1959/6

Degeneration in the central nervous system. The classical histological investigations of central white tracts undergoing Wallerian degeneration suggested that it is a very slow process (CAJAL, 1928; JAKOB, 1912; SKOBLO, 1931). In the present study an appreciable reduction in the concentration of the individual lipid fractions was not observed until 100 days after section of the optic nerve. At this time the various lipid fractions have decreased on the average to a value approximately 55 per cent of the control value except for total cholesterol which decreased only 25 per cent. The concentration of the lipids at 200 days closely approximates to values previously published for central gray matter (LOWRY, ROBERTS, LEINER, WU, FARR and ALBERS, 1954; ROBINS, EYDT and SMITH, 1956). This observation suggests that degeneration in the 200-day optic nerve is nearly complete. The reduction in the concentration of phosphoinositides (Table 5) closely parallels the lipid changes.

The use of DNA as an index of the number of cells indicates that the number in the optic nerve progressively increases until at least 200 days after nerve section. This is essentially the same type of response observed by histological methods in the degenerating posterior columns of the spinal cord (JOSEPH, 1954). However, the proliferative increase in the cord was 2 to 3 times larger than that observed for the optic nerve in the present study. Whether this represents a difference between the cord and optic nerve or a technical difference in comparing histological and chemical results in central white matter is unknown. When the number of cells per mm^3 is calculated as previously described for tibial nerve, a value of $4{\cdot}4 \times 10^5$ cells per mm^3 is obtained. Assuming this is a nearly maximal number of cells implies that factors which limit cellular proliferation (JOSEPH, 1950; ABERCROMBIE and JOHNSON, 1946*b*) apply equally to degenerating peripheral and central nerves.

The finding that the RNA/DNA ratio remains relatively constant during the first 100 days of degeneration in the central nervous system (optic nerve) is in contrast to the increased ratio reported in degenerating peripheral (sciatic) nerve (LOGAN *et al.*, 1952*b*).

Comparison of degeneration in the tibial and optic nerves. There is a marked difference in the *time-course* of Wallerian degeneration in the peripheral and central nervous systems. The rate of degeneration and of the cellular influx is much greater in the peripheral nervous system than in the central nervous system. A decrease in the amount of lipid equivalent to that reported for the 14-day tibial nerve is not reached until after day-100 in the optic nerve. The cellular increase in tibial nerve is maximal at 14 to 25 days and then begins to decrease, whereas in the optic nerve, the cellular increase is still continuing even 200 days after section. The same pattern is obtained for phosphorous fractions and for amino-N in that peaks are reached in tibial nerve at day-14 and not until day-45 to -100 in optic nerve.

In addition to the difference in the rate of Wallerian degeneration, there also appears to be a difference between peripheral and central nerve in the *maximal amount of change* in some of these chemical constituents. The amount of lipid loss is much greater in the tibial than in the optic nerve. The maximal increases in the number of cells and in amino-N in the tibial are somewhat greater than in the optic nerve. Since at 200 days in the optic nerve lipid levels were still decreasing and numbers of cells were still increasing, it cannot be definitely stated that sometime later in the degeneration of the optic nerve the values for these constituents would not have equalled those found in the tibial nerve. It is unlikely that amino-N in the degenerating optic

nerve would ever reach the levels found in the tibial, since there had been only a negligible increase in this constituent from day-45 to day-200. The changes in acid-soluble P fractions are not significantly different in the 2 nerves.

It is of interest to note that there was no difference in the *direction of change* of the levels of any constituent when degenerating tibial and optic nerves were compared. This finding will be contrasted with those for some of the enzymes, where a different direction of change was evident in the 2 nerves (MCCAMAN and ROBINS, 1959).

The reason for the difference in the rate of degeneration in tibial and optic nerves is not clear. A possible explanation may be that the Schwann cell and macrophage of the peripheral nervous system are innately endowed with a greater ability for more rapid increase and more effective removal of myelin than are the glial cell and macrophage of the central nervous system. A second possibility is that there is a more potent humoral stimulus for cellular influx, phagocytosis, and transport in the peripheral than in the central nervous system. A third possibility is that there are physical (physio-chemical) factors that impede the influx of cells into and the transport of myelin out of the degenerated area in the central nervous system. There are other possibilities, and there may be a number of factors (innate cellular differences, a humoral stimulus, and a physical barrier) acting in concert.

Although the reason for this difference in the rate of degeneration is not known, it is tempting to speculate that this different rate is intimately related to the absence of significant functional regeneration in the mammalian central nervous system. It would be of great interest to compare the time-course of Wallerian degeneration in the peripheral and central nervous systems of a species in which central regeneration, with full functional return, occurs readily.

VOL.
5
1959/6

SUMMARY

The quantitative biochemical changes occurring in the tibial and optic nerves (rabbit) undergoing Wallerian degeneration have been determined. The constituents analysed include protein, total lipid, cephalins, lecithins, sphingomyelins, phosphorus-free sphingolipids, total phospholipids, cholesterol, deoxyribonucleic acid (number of cells), total nucleic acid, ribonucleic acid, residual phosphorus (including 'phosphoinositides' and 'phosphoproteins'), acid-soluble inorganic and organic phosphorus, acid-soluble amino-nitrogen, fat-free dry wt, and dry wt per unit volume.

The patterns of lipid depletion were qualitatively similar for the degenerating tibial and optic nerves. There was, however, a marked temporal difference between the two nerves. The rates of lipid decrease were most marked at about 14 and 100 days after section in the tibial and optic nerves, respectively.

A similar temporal difference between the two nerves was observed for the increase in deoxyribonucleic acid (number of cells).

The protein on a fat-free dry weight basis remains relatively constant and the acid-soluble constituents measured increase only slightly during degeneration in both the tibial and optic nerves.

REFERENCES

ABERCROMBIE M. and JOHNSON M. L. (1946*a*) *J. Neurol. Psychiat.* **9,** 113.
ABERCROMBIE M. and JOHNSON M. L. (1946*b*) *J. Anat., Lond.* **80,** 37.
ALBERS R. W. and LOWRY O. H. (1955) *Analyt. Chem.* **27,** 1829.
BRANTE G. (1949) *Acta physiol. scand.* **18,** Suppl. 63, 1.

CAJAL S. R. (1928) *Degeneration and Regeneration of the Nervous System*, Vol. 1 and 2. Oxford University Press, London.
HELLER I. and ELLIOT K. A. C. (1954) *Canad. J. Biochem. Physiol.* **32,** 584.
HOLLINGER D. M. and ROSSITER R. J. (1952) *Biochem. J.* **52,** 659.
HOLLINGER D. M., ROSSITER R. J. and UPMALIS H. (1952) *Biochem. J.* **52,** 652.
HUTCHISON W. C., CROSBIE G. W., MENDES C. B., MCINDOE W. M., CHILDS M. and DAVIDSON J. N. (1956) *Biochim. biophys. acta* **21,** 44.
JAKOB A. V. (1912) *Histologische und Histopathologische Arbeiten uber die Grosshirnrinde von Nissl* **5,** 1.
JOHNSON A. C., MCNABB A. R. and ROSSITER R. J. (1949) *Biochem. J.* **45,** 500.
JOSEPH J. (1950) *Acta anat.* **9,** 279.
JOSEPH J. (1954) *Acta anat.* **21,** 356.
KISSANE J. M. and ROBINS E. (1958) *J. biol. Chem.* **233,** 184.
LEBARON F. N. and FOLCH J. (1956) *J. Neurochem.* **1,** 101.
LOGAN J. E., MANNELL W. A. and ROSSITER R. J. (1952*a*) *Biochem. J.* **52,** 470.
LOGAN J. E., MANNELL W. A. and ROSSITER R. J. (1952*b*) *Biochem. J.* **52,** 482.
LOWRY O. H. (1953) *J. Histochem. Cytochem.* **1,** 420.
LOWRY O. H., ROBERTS N. R., LEINER K. Y., WU M. L. and FARR A. L. (1954) *J. biol. Chem.* **207,** 1.
LOWRY O. H., ROBERTS N. R., LEINER K. Y., WU M. L., FARR A. L. and ALBERS R. W. (1954) *J. biol. Chem.* **207,** 39.
LOWRY O. H., ROSEBROUGH N. J., FARR A. L. and RANDALL R. J. (1951) *J. biol. Chem.* **193,** 265.
LUSE S. and MCCAMAN R. E., *J. biochem. biophys. Cytol.* in press.
MANNELL W. A. (1952) *Canad. J. med. Sci.* **30,** 173.
MCCAMAN R. E. and ROBINS E. (1958) *Neurology*, **8,** Suppl. 1, 86.
MCCAMAN R. E. and ROBINS E. (1959) *J. Neurochem.* **5,** 32.
MCDOUGAL D. B. JR. (1958) *Neurology* **8,** Suppl. 1, 58.
MOTT F. W. and HALLIBURTON W. D. (1901) *Phil. Trans.* B **194,** 437.
NOLL A. (1899) *Hoppe-Seyl. Z.* **27,** 370.
PORCELLATI G. and THOMPSON R. H. S. (1957) *J. Neurochem.* **1,** 340.
ROBINS E. (1957) *Exp. Cell Res.* Suppl. 4, 241.
ROBINS E., EYDT K. M. and SMITH D. E. (1956) *J. biol. Chem.* **220,** 677.
ROBINS E., LOWRY O. H., EYDT K. M. and MCCAMAN R. E. (1956) *J. biol. Chem.* **220,** 661.
ROBINS E., SMITH D. E. and EYDT K. M. (1956) *J. Neurochem.* **1,** 54.
SCHMIDT G. and THANNHAUSER S. J. (1945) *J. biol. Chem.* **161,** 83.
SCHNEIDER W. C. (1945) *J. biol. Chem.* **161,** 293.
SKOBLO M. S. (1931) *Z. ges. Neurol. Psychiat.* **132,** 407.

Journal of Neurochemistry, 1959, Vol. 5, pp. 32 to 42. Pergamon Press Ltd. Printed in Northern Ireland

QUANTITATIVE BIOCHEMICAL STUDIES OF WALLERIAN DEGENERATION IN THE PERIPHERAL AND CENTRAL NERVOUS SYSTEMS—II

TWELVE ENZYMES*

RICHARD E. MCCAMAN†‡ and ELI ROBINS
Departments of Pharmacology and of Psychiatry and Neurology, and the Beaumont-May Institute of Neurology, Washington University School of Medicine, St. Louis, Missouri

(*Received* 26 *February* 1959)

VOL.
5
1959/6

PREVIOUSLY reported quantitative measurements of changes in enzymic activity during Wallerian degeneration in peripheral nerve have been largely confined to two groups of enzymes: those responsible for the destruction (CHOUTEAUX and NACHMANSOHN, 1938; SAWYER, 1946) or synthesis (NACHMANSOHN, JOHN and BERMAN, 1946; BERRY and ROSSITER, 1958) of acetylcholine, and the phosphatases (BODIAN, 1947; HEINZEN, 1947; HOLLINGER, ROSSITER and UPMALIS, 1952; SAMORAJSKI, 1957). The change in the activity of β-glucuronidase during Wallerian degeneration has also been studied (HOLLINGER and ROSSITER, 1952). No quantitative studies have been published regarding enzymic changes during Wallerian degeneration in the white matter of the central nervous system. It was the purpose of the present investigation to study a larger number and variety of enzymes in a degenerating peripheral nerve and to study the same enzymes in a degenerating central white tract. The quantitative histochemical method (LOWRY, 1953; ROBINS, 1957) has been used; its advantages for these studies have been discussed (MCCAMAN and ROBINS, 1959).

MATERIAL AND METHODS

The animals used and the methods of obtaining the frozen-dried, unembedded, and unfixed nerve tissue have been described or referred to in Paper I (MCCAMAN and ROBINS, 1959).

The methods for measuring the enzyme activities were quantitative microchemical spectrophotometric and fluorometric methods. These methods, which require 3 μg or less of brain, dry weight, are described elsewhere: lactic dehydrogenase (ROBINS, ROBERTS, EYDT, LOWRY and SMITH, 1956); aldolase, fumarase and acid phosphatase (LOWRY, ROBERTS, WU, HIXON and CRAWFORD, 1954); purine nucleoside phosphorylase (ROBINS, SMITH and MCCAMAN, 1953); phosphoglucoisomerase (LOWRY, ROBERTS and CHANG, 1956; BUELL, LOWRY, ROBERTS, CHANG and KAPPHAHN, 1958); glucose-6-phosphate dehydrogenase (KUHLMAN and LOWRY, 1956); and isocitric dehydrogenase (LOWRY, ROBERTS and KAPPHAHN, 1957).

During this study a micro method for β-glucuronidase was developed, based on the method suggested by MEAD, SMITH and WILLIAMS (1955). The product of the enzymic reaction, 4-methylumbelliferone§, in alkaline solution gives a strong blue fluorescence. The substrate for the reaction, 4-methylumbelliferone-β-D-glucuronide**, gives a negligible fluorescence under these conditions,

* Supported in part by a grant from the National Multiple Sclerosis Society.

† The data presented in this paper formed part of a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Washington University, 1957.

‡ Present address: The Institute of Psychiatric Research, Indiana University Medical Center, Indianapolis, Indiana.

§ The 4-methylumbelliferone was obtained from Eastman-Kodak Co.

** The 4-methylumbelliferone-β-D-glucuronide was kindly supplied by Professor R. T. Williams.

even when present at 100-fold greater concentration than the product. The following procedure was used to measure the activity of this enzyme. Samples, weighing 1–2 μg (or 1 μl of a 1 : 100 brain homogenate), were placed in tubes 3 mm × 40 mm. Blanks and standards were obtained by using 1 μl of water or 0·05 mM 4-methylumbelliferone. Five μl of buffer-substrate (0·5 mM- 4-methylumbelliferone-β-D-glucuronide in 0·1 M-acetate buffer, pH 3·9) were added to the tubes in an ice bath (0–4°). The tubes were capped with Parafilm, mixed, and placed in a 38° water bath. (During the incubation a comparable number of Pyrex tubes, 10 × 75 mm, Corning No. 9820, were filled with 1 ml of 0·1 M-2-amino-2-methyl-1-propanol buffer, pH 10·4.) After exactly 30 min of incubation the tubes were returned to the ice bath and a 3–4 μl aliquot was removed and added to the buffer in the Pyrex tube. The samples were then read in the Farrand fluorometer, model A. The exciting light was the 365 mμ Hg line, isolated with the Corning glass filter No. 5860. The measured emission was that transmitted by the combination of filters No. 5543 and 3387. This combination gives transmission between 460 and 480 mμ.

Optimal substrate concentration during incubation is 0·5 to 0·7 mM. Optimal pH is between 3·9 and 4·2 in acetate buffer. The reaction was linear with time for at least 10 hr with 1 μg, wet wt, of brain. The β-glucuronidase activity of fresh rabbit brain homogenate is 2 m-moles per kg, wet wt, per hr.

L-Leucylalanine is used as the substrate for the peptidase measurement (McCaman and Lowry, to be published). The buffer-substrate reagent used contains α-ketoglutarate, DPNH (reduced diphosphopyridinenucleotide), and purified glutamic–alanine transaminase and lactic dehydrogenase. Thus, the alanine liberated by the action of peptidase is quantitatively converted to lactic acid and a stoichiometric amount of DPN^+ (diphosphopyridinenucleotide). The DPN^+ is measured fluorometrically (Lowry, Roberts and Kapphahn, 1957).

The activity of α-glycerol phosphate dehydrogenase was measured by the method of McDougal and Schimke (to be published). The incubation mixture contained 30 μmoles nicotinamide, 1·0 mg bovine plasma albumin, 1·0 μmole DPNH, and 2·4 μmoles dihydroxyacetonephosphate per ml of 0·1 M-phosphate buffer, pH 7·5. The DPN^+ formed was measured fluorometrically as above.

The substrate used to measure the acitivity of β-galactosidase was 4-methylumbelliferone-β-D-galactoside.* The 4-methylumbelliferone liberated during the reaction was measured fluorometrically as described under the method for β-glucuronidase. Incubation was carried out in 0·1 M-acetate buffer, pH 3·7, containing the substrate at a concentration of 1·1 M. Under these conditions the activity of rabbit brain homogenate is 13 m-moles per kg, wet wt, per hr.

The standard errors of the mean listed in Tables 1–5 are derived from multiple determinations from several nerves. Thus, they do not give an index of either methodological variability or biological variability but rather a composite of both. A *P*-value of 0·01 or less was accepted as being statistically significant.

RESULTS

Comparison of control tibial and optic nerves

There are slight to marked differences in the levels of the 12 enzymes studied between tibial and optic nerves. For each enzyme the optic nerve has a higher level of activity, based on fat-free dry wt, than does the tivial nerve (Table 1). Activities of the different enzymes are from 10 per cent to over 700 per cent greater in the optic than in the tibial nerve. These higher enzymic activities in optic nerve probably indicate a higher rate of metabolic events, which tends to confirm the same conclusion reached in Paper I (McCaman and Robins, 1959), based on the findings of higher levels of acid-soluble phosphorus and amino-N fractions in the optic nerve.

Degenerating tibial and optic nerves

The enzymic activities were determined directly as a function of dry wt. However, the loss of lipids, which amounts to more than 50 per cent of the dry wt, results in a marked change in the total dry wt of the nerves during degeneration. Therefore,

* Kindly supplied by Dr. Melvin Cohn.

the results are recorded in terms of the fat-free dry wt (most of which is of course protein). The changes in the fat-free dry wt of the nerves have been reported in the first paper of this series (MCCAMAN and ROBINS, 1959).

TABLE 1.—RATIOS OF ENZYME ACTIVITIES BETWEEN NORMAL OPTIC AND TIBIAL NERVES

	Ratio* Optic / Tibial		Ratio* Optic / Tibial
Phosphoglucoisomerase	7·6	Acid phosphatase	2·0
Aldolase	4·1	β-Galactosidase	1·6
Lactic dehydrogenase	3·1	Fumarase	1·4
Peptidase	3·0	β-Glucuronidase	1·2
Purine nucleoside phosphorylase	3·0	Glucose-6-PO_4 dehydrogenase	1·2
Isocitric dehydrogenase	2·6	α-Glycerol-PO_4 dehydrogenase	1·1

* The data from which these ratios were calculated will be found in Tables 2 and 3, in the first column.

VOL. 5 1959/6

Tibial nerve (Table 2). Of the 12 enzymes studied, there were 7 (Nos. 1–7, Table 2) which showed an overall increase in acitivity at some period during 100 days of degeneration, 4 (Nos. 8–11) which showed a decrease, and 1 (No. 12) which showed negligible change. Each of the enzymes which increased reached a maximum at 14 days and thereafter decreased somewhat, although in all but two instances (peptidase and fumarase) the enzymic activities at 100 days were still above the

TABLE 2.—ENZYMIC ACTIVITIES OF DEGENERATING TIBIAL NERVES

All values, except as noted, are expressed as moles of substrate converted/kg fat-free dry wt/hr ± standard error of the mean, and represent the mean of 3–5 determinations on each nerve from 3–6 animals for the control and 2 animals for the degenerating tissue at each time.

Enzyme	Days of degeneration			
	0	14	45	100
1. β-Glucuronidase†	18·6 ± 0·9*	380 ± 15	131 ± 4*	57·4 ± 1·6*
2. β-Galactosidase†	29 ± 1*	440 ± 50	310 ± 20	90 ± 10*
3. Purine nucleoside phosphorylase	0·26 ± 0·02*	0·82 ± 0·06	0·71 ± 0·07	0·52 ± 0·03*
4. Acid phosphatase	0·48 ± 0·03*	1·69 ± 0·05	1·62 ± 0·21	1·44 ± 0·14
5. Peptidase	5·5 ± 0·3*	14·0 ± 1·8	6·2 ± 0·4*	4·7 ± 0·9*
6. Isocitric dehydrogenase	0·88 ± 0·03*	1·73 ± 0·12	1·71 ± 0·13	1·07 ± 0·08*
7. Fumarase	3·73 ± 0·11*	4·90 ± 0·33	2·89 ± 0·16*	1·63 ± 0·21*
8. α-Glycerol-PO_4 dehydrogenase	1·74 ± 0·10	0·23 ± 0·03*	0·09 ± 0·01*	0·04 ± 0·01*
9. Aldolase	1·32 ± 0·06	1·05 ± 0·02*	0·54 ± 0·04*	0·55 ± 0·03*
10. Lactic dehydrogenase	8·95 ± 0·39	8·55 ± 0·51	8·10 ± 0·39	5·75 ± 0·67*
11. Glucose-6-PO_4 dehydrogenase	1·81 ± 0·13	1·96 ± 0·19	1·23 ± 0·11*	1·42 ± 0·12
12. Phosphoglucoisomerase	18·7 ± 0·6	19·5 ± 0·4	20·2 ± 1·7	16·1 ± 0·5

* Significantly different ($P < 0·01$) from the highest level of activity for each enzyme, which is underlined in the table.

† Millimoles of substrate converted kg fat-free dry wt/hr.

control level for each. The greatest percentage increase was shown by β-glucuronidase (Table 4), which reached a level 20-fold greater than in the control. HOLLINGER and ROSSITER (1952) have previously reported a striking increase in β-glucuronidase activity in the degenerating sciatic nerve of the cat. The observed increase in acid phosphatase activity during degeneration of the tibial nerve (Table 2) confirms the previous findings of BODIAN (1947), HOLLINGER, ROSSITER and UPMALIS (1952), and SAMORAJSKI (1957). Using histochemical methods, WOLF, KABAT and NEWMAN (1943) detected the presence of acid phosphatase in the axons of normal peripheral nerves. A slight activity was also exhibited by the Schwann cells, primarily in the nuclei. The increase during degeneration, however, appears to be associated with the increase in Schwann cells. SAMORAJSKI (1957) obtained a very good correlation between the increase in acid phosphatase activity and the increase in Schwann cells at 8 and 13 days after transection of the sciatic nerve; the correlation at a later time (54 days), however, was not as good.

The enzymes which decreased in activity showed a differing pattern in time. The enzyme which showed the most striking decrease was α-glycerol phosphate dehydrogenase, which had decreased in 14 days to only 13 per cent and in 100 days to only 2 per cent of its control value (Table 4). Aldolase and glucose-6-phosphate dehydrogenase showed most marked decreases at 45 days. Lactic dehydrogenase showed an appreciable decrease only at 100 days.

Phosphoglucoisomerase showed a relatively negligible change during the entire 100 days.

The glycolytic enzymes did not show a parallelism during the course of degeneration. The 2 enzymes of the citric acid cycle were parallel in their changes, except at 45 days, when fumarase showed a significant decrease, whereas isocitric dehydrogenase remained at the same level.

Optic nerve (Table 3). Of the 12 enzymes studied, there were 6 (Nos. 1–6, Table 3) which showed an overall increase in activity during the 100 days of degeneration, 3 (Nos. 7–9) which showed a decrease, and 3 (Nos. 10–12) which showed no statistically significant change. Each of the enzymes which increased did not reach a maximum before day-45. Two (Nos. 1 and 2) reached a maximum on this day and the other 4 did not reach a maximum until day-100. In each instance, except one (No. 5), there was at least a 40 per cent increase by day-14 (Table 4). The overall pattern, therefore, is one of an appreciable increase by day-14 which continued, reaching a maximum at day-45 or day-100. As in the tibial nerve, β-glucuronidase showed the greatest percentage increase in activity and β-galactosidase the next greatest increase.

Each of the 3 enzymes which showed a decrease in activity showed its most marked change by day-14, although the lowest value was reached on day-45 or day-100. Aldolase and phosphoglucoisomerase showed a substantially greater decrease than did lactic dehydrogenase.

Isocitric dehydrogenase, fumarase and glucose-6-phosphate dehydrogenase showed only negligible changes from control values. Because of the dispersion of the data for the latter enzyme at day-45, the apparent increase is not statistically significant.

In the optic nerve the glycolytic enzymes changed in the same direction even though there were some quantitative differences. Similarly, the 2 citric acid cycle enzymes (fumarase and isocitric dehydrogenase) showed a parallelism throughout the period of degeneration studied. Each of these enzymes showed only a negligible change.

TABLE 3.—ENZYMIC ACTIVITIES OF DEGENERATING OPTIC NERVES

All values, except as noted, are expressed as moles of substrate converted/kg fat-free dry wt/hr ± standard error of the mean, and represent the mean of 3–5 determinations on each nerve from 3–6 animals for the control and 2 animals for the degenerating tissue at each time.

Enzyme	Days of degeneration			
	0	14	45	100
1. β-Glucuronidase†	21·6 ± 1·0*	85 ± 4*	183 ± 6	120 ± 8*
2. β-Galactosidase†	46 ± 4*	98 ± 7*	277 ± 32	136 ± 9*
3. Purine nucleoside phosphorylase	0·79 ± 0·04*	1·38 ± 0·08	1·23 ± 0·11	1·54 ± 0·12
4. Acid phosphatase	0·95 ± 0·05*	1·33 ± 0·04*	1·61 ± 0·12	1·62 ± 0·08
5. Peptidase	16·2 ± 0·8*	20·3 ± 2·3*	30·9 ± 5·0	35·6 ± 4·0
6. α-Glycerol-PO_4 dehydrogenase	1·98 ± 0·13*	3·17 ± 0·28	2·77 ± 0·26	3·58 ± 0·34
7. Aldolase	5·46 ± 0·26	2·82 ± 0·18*	1·59 ± 0·13*	2·08 ± 0·10*
8. Phosphoglucoisomerase	142 ± 7	58 ± 3*	43 ± 2*	38 ± 3*
9. Lactic dehydrogenase	27·5 ± 2·3	20·3 ± 2·0	21·9 ± 1·7	16·8 ± 2·8*
10. Isocitric dehydrogenase	2·24 ± 0·18	1·96 ± 0·18	2·59 ± 0·18	2·62 ± 0·34
11. Fumarase	5·18 ± 0·18	5·53 ± 0·28	5·11 ± 0·29	5·36 ± 0·20
12. Glucose-6-PO_4 dehydrogenase	2·11 ± 0·23	1·86 ± 0·30	2·95 ± 0·50	2·42 ± 0·44

* Significantly different ($P < 0·01$) from the highest level of activity of each enzyme, which is underlined in the table.

† Millimoles of substrate converted kg fat-free dry wt/hr.

Comparison of degenerating tibial and optic nerves. There were 7 enzymes which behaved in a qualitatively similar fashion in the degenerating tibial and optic nerves. Five of these (Nos. 1–5, Table 4) showed an increase. There were 2 differences between the nerves for enzymes which increased. (a) Maximal levels of activity in the tibial nerve were reached at 14 days, whereas in the optic nerve maximal levels were reached only after 45 or 100 days. This greater rate of increase for these enzymes in tibial nerve correlates well with the greater rapidity of nerve degeneration and myelin removal in tibial nerve (MCCAMAN and ROBINS, 1959). (b) Greater percentage increases of activity were uniformly found for each enzyme in the tibial nerve than in the optic nerve. These differences ranged from over twice as much of an increase for β-glucuronidase and β-galactosidase to approximately a 25 per cent greater increase for peptidase.

The 2 remaining enzymes which behaved in a qualitatively similar fashion in the tibial and optic nerves showed a decrease in activity (Nos. 6 and 7, Table 4). Aldolase decreased less abruptly in tibial nerve than in optic nerve. In tibial nerve aldolase had decreased to 80 per cent of its original value at 14 days, whereas in optic nerve it had decreased to 52 per cent of its original value. At 45 and 100 days the amount of decrease in both nerves was more nearly similar. Lactic dehydrogenase also decreased less abruptly in tibial than in the optic nerve, although at 100 days the percentage decrease was almost equal in the 2 nerves.

There were 5 enzymes that behaved differently in the 2 nerves (Nos. 8–12, Table 4). One enzyme, α-glycerol phosphate dehydrogenase, showed a profound and abrupt

VOL. 5 1959/6

TABLE 4.—A COMPARISON OF PERCENTAGE CHANGES IN ENZYMIC ACTIVITIES IN DEGENERATING TIBIAL AND OPTIC NERVES

These percentages were calculated from the data in Tables 2 and 3, the highest activity for each enzyme in each nerve being set at 100 per cent.

Enzyme	Nerve Tibial (T) Optic (O)	Days of degeneration			
		0	14	45	100
1. β-Glucuronidase	T	5	100	34	16
	O	12	47	100	66
2. β-Galactosidase	T	7	100	70	20
	O	17	35	100	49
3. Purine nucleoside phosphorylase	T	31	100	86	63
	O	51	90	80	100
4. Acid phosphatase	T	28	100	96	85
	O	59	82	99	100
5. Peptidase	T	39	100	44	33
	O	46	57	87	100
6. Aldolase	T	100	80	41	42
	O	100	52	29	38
7. Lactic dehydrogenase	T	100	96	91	64
	O	100	74	80	61
8. α-Glycerol-PO_4 dehydrogenase	T	100	13	5	2
	O	55	89	78	100
9. Glucose-6-PO_4 dehydrogenase	T	93	100	63	72
	O	72	63	100	82
10. Fumarase	T	76	100	59	34
	O	94	100	93	96
11. Isocitric dehydrogenase	T	51	100	99	62
	O	86	74	98	100
12. Phosphoglucoisomerase	T	93	96	100	80
	O	100	41	30	27

decrease in tibial nerve, decreasing to only 13 per cent of its original level in 14 days and to 2 per cent by day-100, whereas in optic nerve it showed a 40–80 per cent increase in activity. Glucose-6-phosphate dehydrogenase also showed a decrease in the tibial nerve, whereas in optic nerve it did not change appreciably. In tibial nerve isocitric dehydrogenase and fumarase showed a significant increase followed by a significant decrease, whereas in optic nerve neither changed appreciably. Finally, phosphoglucoisomerase showed no change in the tibial nerve but a marked decrease in the optic nerve to only 27 per cent of its original activity in 100 days.

DISCUSSION

Comparison of normal tibial and optic nerves. Each of the 12 enzymes is richer in the optic nerve than in the tibial nerve. Is this finding generally true of central tracts when compared with peripheral nerve? There have been studies of 6 of these enzymes, fumarase, isocitric dehydrogenase, phosphoglucoisomerase, aldolase, acid phosphatase, and glucose-6-phosphate dehydrogenase, reported in central white matter other than that of optic nerve in the rabbit (LOWRY, ROBERTS, LEINER, WU, FARR and ALBERS, 1954; STROMINGER and LOWRY, 1955; BUELL *et al.*, 1958; MCDOUGAL, 1958). For

5 of the 6 enzymes (the exception is glucose-6-phosphate dehydrogenase) the levels of activity reported in from 1 to 7 additional central tracts are higher than that of the tibial nerve; this result suggests that the difference may be a general one. It has been shown that lactic dehydrogenase and phosphoglucoisomerase are unusually rich in portions of the rabbit optic pathway (STROMINGER and LOWRY, 1955; BUELL *et al.*, 1958). This finding does not completely account for the higher levels of these enzymes in optic nerve, since in each of 7 other central tracts so far studied phosphoglucoisomerase is richer than in tibial nerve, and in the one other white tract (alveus) in which lactic dehydrogenase has been measured it is higher than in tibial nerve. However, until a very different peripheral nerve, e.g. the almost completely non-myelinated vagus nerve, is also studied, it is premature to conclude that the difference for these 5 enzymes is a general one between all central tracts and peripheral nerves. In contrast with the findings for the first 5 enzymes, glucose-6-phosphate dehydrogenase, which has been measured in 7 other central tracts, is less rich in one (*mammillothalamic*) and equally rich in another (*alveus of Ammon's horn*) when compared with tibial nerve.

Relationship of enzymic changes to morphological changes in degenerating nerves. Morphological changes as studied with the electron microscope will be reported elsewhere (LUSE and MCCAMAN, in press). Some of the findings relevant to the present report are briefly summarized. In the tibial nerve, at 14 days, axonal degeneration is almost complete; severe myelin destruction is present; there is a very high level of cellular proliferation of Schwann cells and macrophages; and phagocytosis is occurring. In the optic nerve at 14 days, axonal degeneration is fairly far advanced but not completed; there are only physical changes in the myelin; there is minimal cellular proliferation with only rare macrophages; and no phagocytosis is occurring.

At 45 days in the tibial nerve all of the axonal debris is gone; there is a relatively small amount of myelin debris remaining; and the Schwann cells are about the same as at 14 days, but there are fewer macrophages. In the optic nerve there is still some axonal debris remaining; the myelin is less dense, but still shows little evidence of chemical dissolution or of being phagocytized; and there is a slight proliferation of astrocytes, but macrophages are still rare.

At 100 days in the tibial nerve almost all of the myelin debris is gone; cords of Schwann cells are prominent; there is a further decrease in the number of macrophages; and fibroblasts are prominent. In the optic nerve, for the first time marked myelin destruction and phagocytosis are evident; a glial (astrocytic) scar is present; and there is marked increase in the number of macrophages (microglia).

In the tibial nerve, the maximal increases in enzymes 1–7 (Table 2) occur at day 14 with a subsequent decrease at later times. Since the time-course of cellular influx and proliferation is temporally similar (MCCAMAN and ROBINS, 1959), these increases are presumably due to the influx and proliferation of Schwann cells and macrophages. This interpretation, and some that follow, makes 2 assumptions for which there is at present no direct evidence: (1) that no small molecule activators and inhibitors are released during degeneration; and (2) that degeneration of axons, myelin, or any other constituent does not release 'bound' enzyme which has been present but inactive in the normal nerve. The first assumption is probably justified because the enzyme incubations were done at tissue dilutions of 5000–25,000, so that any potential inhibitor or activator was probably diluted to an ineffective concentration. It is possible to test this assumption by adding degenerating nerve or an extract of such

VOL.
5
1959/6

nerve to normal nerve. The justification for the second assumption is based on previous studies of enzymic activity in brain tissue after varying post-mortem intervals (SMITH *et al.*, 1957; ROBINS *et al.*, 1958). In these investigations it was shown that 8 of the enzymes reported in the present study did not show significant changes in autolying brain tissue, including central white matter, for as long as 6 hr after death, and for as long as 24 hr in a homogenate left at room temperature. These findings suggest that it is unlikely that 'bound' enzyme was released by the degenerating nerves.

The results of these 2 assumptions are that axonal and myelin destruction is assumed either not to affect a given enzyme or to cause a decrease in its actual amount as well as in its activity; and that any increase in enzymic activity reflects an increase in the actual amount of enzyme, resulting either from an increase in the number of cells containing this enzyme (glial or Schwann or macrophages) or from an increased amount of enzyme because of the changed functional state of the cells, e.g. hypertrophic Schwann cells with fibril formation (LUSE and MCCAMAN, in press) and phagocytic macrophages. According to the foregoing reasoning, any change in the activity of an enzyme is the sum of its loss owing to axonal or myelin destruction and of its increase owing to the influx or change in functional state of cellular elements. Obviously, if an enzyme is not localized in a given element its increase or destruction will not affect the level of that particular enzyme.

It is evident, however, that the increases in the various enzymic activities do not necessarily quantitatively parallel the increases in numbers of cells. This lack of parallelism is not unexpected since axons and the protein moiety of myelin may contain these enzymes, so that their level is a balance of increase and destruction.

In the optic nerve, the finding of the maximal increases in enzymes 1–6 (Table 3) at day-45 or day-100, suggests that these increases are due to the influx and proliferation of astrocytes and macrophages (microglia), since the cellular increase is still continuing at these times. There is a suggestion from the electron microscopic evidence (LUSE and MCCAMAN, in press) that there is only a slight increase in the numbers of oligodendroglia. They may, therefore, contribute quantitatively only very slightly to these changes. It is evident, however, that the increases in the various enzymic activities do not quantitatively parallel the increases in the number of cells (MCCAMAN and ROBINS, 1959), which show increases of 33, 62 and 81 per cent at days 14, 45 and 100, respectively. In fact, the significant decreases in β-glucuronidase and β-galactosidase at 100 days are inconsistent with the continuing increase in the cells, unless a differing functional state of the cells, or some other process, is invoked as an explanation.

Of the 4 enzymes which decrease in the degenerating tibial nerve (Nos. 8–11, Table 2), there is only one, α-glycerol phosphate dehydrogenase, which shows a marked fall at 14 days. This suggests that this enzyme may be localized primarily in the axis cylinders of peripheral nerve. This observation is similar to that reported for 'true' cholinesterase (SAWYER, 1946), choline acetylase (NACHMANSOHN, JOHN and BERMAN, 1946), and alkaline phosphatase (HOLLINGER, ROSSITER and UPMALIS, 1952) in degenerating peripheral nerves. The remainder of the enzymes show their most marked decreases later, so that it is difficult to interpret the findings concerning them in histological terms. It is possible that they are present in axons and/or myelin as well as in the Schwann cells or macrophages, so that these findings are a balance of increase and destruction of the enzyme.

Of the 3 enzymes which decrease in the degenerating optic nerve, 2, aldolase and phosphoglucoisomerase, show a marked decrease at 14 days with a further less-marked decrease subsequently. Lactic dehydrogenase shows a less marked decrease which changes relatively little subsequently. These findings may be interpreted as indicating an axonal localization for aldolase and phosphoglucoisomerase in the optic nerve. It has been shown that phosphoglucoisomerase has a relatively high activity in portions of the rabbit optic pathway when compared with other central white tracts (LOWRY, 1955; STROMINGER and LOWRY, 1955). A subsequent study indicated that the cells of origin of the optic nerve in the ganglion cell layer of the retina have very high levels of phosphoglucoisomerase activity (LOWRY, ROBERTS and LEWIS, 1956). The high activity of phosphoglucoisomerase in the optic nerve may be a reflection of the enzymic character of its cell of origin, and when it is separated from this cell there is a rapid decrease in its phosphoglucoisomerase activity.

In the tibial nerve isocitric dehydrogenase increased at day 14, whereas in the optic nerve it showed a negligible change at each time after section. Since the macrophages of the peripheral and central nervous systems are probably identical, this finding suggests that Schwann cells are rich in isocitric dehydrogenase and astroyctes are not, or that axons and myelin in the central nervous system differ in their content of this enzyme from similar structures in the peripheral nervous system.

Similarly, the increase in α-glycerol phosphate dehydrogenase in the degenerating optic nerve and its profound decrease in the tibial nerve suggest a differing localization or function for this enzyme in the central and peripheral nervous systems. As suggested above, it may be preferentially localized in peripheral axis cylinders. KENNEDY (1953) has shown that L-α-glycerol phosphate is an obligate intermediate in the biosynthesis of the glycerophosphatides. In addition, BARANOWSKI (1949) has shown that the equilibrium constant is heavily in favour of the formation of L-α-glycerophosphate from dihydroxyacetonephosphate in the presence of the dehydrogenase. Glycerokinase, the alternate mechanism for forming glycerol phosphate, has not yet been shown to occur in nervous tissue. The decreased concentration of the glycerophosphatides in the degenerating peripheral nerve may result in part from a reduction in biosynthetic enzyme activity. BERRY, MCPHERSON and ROSSITER (1958) have recently shown a 60–70 per cent decrease in choline phosphokinase activity in peripheral nerve, which tends to support the interpretation of decreased lipid biosynthesis in degenerating peripheral nerve. If α-glycerol phosphate dehydrogenase is predominately localized in the peripheral axon, this would provide a mechanism for the axonal regulation of myelogenesis by the Schwann cell. For, without the presence of axonal formed L-α-glycerophosphate, the Schwann cell would be limited in lipid biosynthesis and therefore in myelin sheath formation. Supporting evidence (MCCAMAN, to be published) for such speculation is found in the very low enzymic activity of α-glycerol phosphate dehydrogenase in the abdominal vagus nerve of the rabbit (this portion of the vagus nerve is predominately non-myelinated but nevertheless rich in Schwann cells and axons). The assumption made is that the axons of this portion of the vagus nerve contain very little dehydrogenase and, therefore, no myelin sheath is formed. In the central nervous system, on the other hand, the apparent localization of the dehydrogenase in the glial elements requires the postulation of a different mechanism for axonal control over the myelin sheath formation by the satellite cell.

VOL.
5
1959/6

SUMMARY

Quantitative microchemical methods have been applied to the study of 12 enzymes and the changes in their activities during Wallerian degeneration in the tibial and optic nerves of the rabbit at 14, 45, and 100 days after transection of the nerves.

There was an overall qualitative similarity in the enzymic changes in degenerating tibial nerve with those in degenerating optic nerve for 7 of the 12 enzymes: 5 increased (β-galactosidase, purine nucleoside phosphorylase, peptidase, β-glucuronidase, and acid phosphatase); and 2 decreased (aldolase and lactic dehydrogenase).

There were striking temporal differences, however, between the enzymes which increased or decreased in both nerves. In the tibial nerve the 5 enzymes which showed an increase in activity had reached a peak at day-14, whereas in the optic nerve they did not reach a peak until 45 or 100 days after section. For the enzymes which decreased in activity, there was a more abrupt drop, as measured at day-14, in the optic than in the tibial nerve.

The changes during degeneration in the activities of the remaining enzymes were not even qualitatively similar for the tibial and optic nerves. These included: for α-glycerol phosphate dehydrogenase, an increase in optic nerve in contrast to a marked decrease in tibial nerve; for phosphoglucoisomerase, a decrease in optic nerve as compared to relatively constant values in tibial nerve; for isocitric dehydrogenase, an increase in tibial nerve as compared to no change in optic nerve; for fumarase, an increase followed in a later period by a decrease in tibial nerve as compared to no change in optic nerve; and for glucose-6-phosphate dehydrogenase, a decrease in tibial nerve as compared to relatively small fluctuations in the optic nerve.

REFERENCES

BARANOWSKI T. (1949) *J. biol. Chem.* **180,** 535.

BERRY J. F., MCPHERSON C. F. and ROSSITER R. J. (1958) *J. Neurochem.* **3,** 65.

BERRY J. F. and ROSSITER R. J. (1958) *J. Neurochem.* **3,** 59.

BODIAN D. (1947) *Symp. Soc. exp. Biol.* **1,** 170.

BUELL M. V., LOWRY O. H., ROBERTS N. R., CHANG M.-L. W. and KAPPHAHN J. I. (1958) *J. biol. Chem.* **232,** 979.

CHOUTEAUX R. and NACHMANSOHN D. (1938) *Nature, Lond.* **142,** 481.

HEINZEN B. (1947) *Anat. Rec.* **98,** 193.

HOLLINGER D. M. and ROSSITER R. J. (1952) *Biochem. J.* **52,** 659.

HOLLINGER D. M., ROSSITER R. J. and UPMALIS H. (1952) *Biochem. J.* **52,** 652.

KENNEDY E. P. (1953) *J. biol. Chem.* **201,** 399.

KUHLMAN R. E. and LOWRY O. H. (1956) *J. Neurochem.* **1,** 173.

LOWRY O. H. (1953) *J. Histochem. Cytochem.* **1,** 420.

LOWRY O. H. (1955) *Biochemistry of the Developing Nervous System* p. 350. Academic Press, New York.

LOWRY O. H., ROBERTS N. R. and CHANG M.-L. (1956) *J. biol. Chem.* **222,** 97.

LOWRY O. H., ROBERTS N. R. and KAPPHAHN J. I. (1957) *J. biol. Chem.* **224,** 1047.

LOWRY O. H., ROBERTS N. R., LEINER K. Y., WU M.-L., FARR A. L. and ALBERS R. W. (1954) *J. biol. Chem.* **207,** 39.

LOWRY O. H., ROBERTS N. R. and LEWIS C. (1956) *J. biol. Chem.* **220,** 879.

LOWRY O. H., ROBERTS N. R., WU M.-L., HIXON W. S. and CRAWFORD E. J. (1954) *J. biol. Chem.* **207,** 19.

LUSE S. and MCCAMAN R. E. *J. biophys. biochem. Cytol.*, in press.

MAGEE W. L. and ROSSITER R. J. (1954) *Biochem. J.* **58,** 243.

MCCAMAN R. E. and ROBINS E. (1959) *J. Neurochem.* **5,** 18.

MCDOUGAL D. B. (1958) *Neurology* **8**: Suppl. 1, 58.

MEAD J. A. R., SMITH J. N. and WILLIAMS R. T. (1955) *Biochem. J.* **61,** 569.
NACHMANSOHN D., JOHN H. M. and BERMAN M. (1946) *J. biol. Chem.* **163,** 475.
ROBINS E. (1957) *Exp. Cell Res.* Suppl. 4, 241.
ROBINS E., ROBERTS N. R., EYDT K. M., LOWRY O. H. and SMITH D. E. (1956) *J. biol. Chem.* **218,** 897.
ROBINS E., SMITH D. E., DAESCH G. E. and PAYNE K. E. (1958) *J. Neurochem.* **3,** 19.
ROBINS E., SMITH D. E. and MCCAMAN R. E. (1953) *J. biol. Chem.* **204,** 927.
SAMORAJSKI T. (1957) *J. Histochem. Cytochem.* **5,** 15.
SAWYER C. H. (1946) *Amer. J. Physiol.* **146,** 246.
SMITH D. E., ROBINS E. EYDT K. M. and DAESCH G. E. (1957) *Lab. Invest.* **6,** 447.
STROMINGER J. L. and LOWRY O. H. (1955) *J. biol. Chem.* **213,** 635.
WOLF A., KABAT E. A. and NEWMAN W. (1943) *Amer. J. Path.* **19,** 423.

VOL.
5
1959/6

Journal of Neurochemistry, 1959, Vol. 5, pp. 43 to 49. Pergamon Press Ltd. Printed in Northern Ireland

RIBONUCLEIC ACID CHANGES IN THE MOTONEURONS OF THE FROG DURING AXON REGENERATION*

J.-E. EDSTRÖM
Department of Histology, Medicinargatan 5, The University of Gothenburg, Sweden

(*Received* 24 *April* 1959)

IT HAS been shown that marked changes in the basophilic and ultraviolet absorbing material, which is known to contain ribonucleic acid (RNA), occur in the cell bodies of motoneurons during the regeneration of parts of their axons (NISSL, 1892; GERSH and BODIAN, 1943; HYDÉN, 1943). Many authors agree that there is a loss of RNA in the early phases of this process, but the findings of BRATTGÅRD, EDSTRÖM and HYDÉN (1957) show that this view may not always be correct. Their microchemical measurements of RNA during axon regeneration in hypoglossal neurons of the rabbit do not show any decrease but on the contrary an increase in RNA content. It is, however, possible that the case studied is an exception to the rule. In view of the interest in this question it would be valuable to study this problem also in other kinds of neurons which are regenerating axons.

In the present work RNA and volume changes were studied in the cell bodies of regenerating spinal motoneurons of the frog *Rana temporaria*. The results differed in some respects from those obtained on the rabbit, but again in this case the important change was found to be an RNA increase.

MATERIALS AND METHODS

Members of both sexes of the species *Rana temporaria* in the size range of 20–30 g were used. They were collected during the mating period in a nearby pond and were operated on shortly afterwards. Altogether 17 animals were used for histological and functional investigations; 9 of them were also used for microchemical measurements.

The animals were anaesthetized with ether, the left sciatic nerve was exposed in the middle of the thigh and the nerve was crushed with forceps. The wound was then closed with a clip and the amphibia were kept at room temperature (about 20°) in a humid environment until they were killed for investigation. Histological investigations were carried out on material having the following regeneration times: 4, 6, 8, 10, 12, 14, 18, 21, 26, 31, 42, 51, 66, 81, 100, 122 and 151 days. Microchemical analyses were made on the following of these: 4, 8, 12, 14, 18, 31, 42, 66 and 151 days. The frogs were decapitated, eviscerated and skinned, after which the spinal canal was opened and the cord removed. It was fixed for 90 min *in toto* in Carnoy's fluid and then embedded in solid paraffin after passing through absolute ethanol and benzene.

Absence of reactions to sensory stimuli and of active movements in the distal parts of the left leg was taken as criteria for the success of the nerve operations. The extent of functional regeneration was measured in the animals after the spinal cords had been removed, by stimulating the sciatic nerves electrically.

The spinal cords were sectioned from the caudal end and sections for analysis were made at a position about 500 μ cranial to the site where the ventrolateral cell group was first discernible. Two sections, each 70 μ thick, were normally used for RNA analyses. Stained control sections were taken below, between and above these sections; these stained sections were made alternately 7 μ and 70 μ thick. They were stained, like the other material, with methylene blue at pH 4·6 according

* The investigation was supported by a grant from the Swedish Medical Research Council.

to PISCHINGER (1926) and RNA was analysed according to EDSTRÖM (1958). Briefly the principle of this method is as follows: individual cells are dissected out of fixed tissue with the aid of a micromanipulator; RNA is then quantitatively collected by repeated extraction with a solution containing ribonuclease; after which the extracted RNA is dissolved in circular drops which are investigated by a photographic-photometric procedure in monochromatic ultraviolet light. The standard error of this method is about ±5 per cent. The RNA analyses on the contralateral, non-operated side served as controls. In this way the influence of variation between animals could be minimized. From each animal 15–20 cells were measured on both sides in groups of 3–4 cells. Altogether 315 cells were analysed in 85 determinations.

The volume of the cells, which had been dissected from the tissue, was also measured. The most exact method at present for volume determination of nerve cells is optical sectioning and three-dimensional volume reconstruction (MICKLEWRIGHT, KURNICK and HODES, 1953) which is particularly well suited for dissected-out, unstained cells. Since it is, however, too time-consuming for a large number of cells, a simpler method was used. The dissected cells were placed in glycerol in the chamber used for the RNA determinations. Camera lucida drawings were made of the optical section of largest area and this area was determined by planimetry. The cell thickness was then determined as the distance between the upper and lower focal planes, which just touched the cell, when using an immersion objective of N.A. 1·25. The formula for the calculation of the volume is: $V = Shn_1 \times 2f/3n_2$, where V = the volume in μ^3, S = the area of the optical section in μ^2, h = the cell thickness in μ, n_1 and n_2 = the refractive indices of glycerol and the immersion oil and f = an empirical factor, 0·924, which corrects a systematic error inherent in the formula. Simplified the formula may be written: $V = 0{\cdot}6\,Sh$.

The value for f was derived by comparing the volumes of 56 cells obtained by three-dimensonal reconstruction and by the simpler method. The results showed that the simplified procedure gave a mean value of 108·2 per cent of the volume determined by optical sectioning, with a standard deviation of ±9·5 per cent. By the use of f the systematic error is therefore reduced. The accuracy of this procedure, though not high, is sufficient for the purpose considering the large variation between different cells and that 15–20 cells are measured from each side of the spinal cord.

RESULTS

Functional regeneration. The first signs of functional regeneration were obtained after 42 days, when the muscles in the leg showed faint response to stimulation. At 51 days the tarsal muscles were reinnervated, as were the most distal muscles after 66 days, although the response on stimulation was considerably weaker than on the other side. The strength of the response to stimulation gradually increased so that after 122 days only a slight difference was noted between the sides and after 151 days none at all.

Histological picture. In agreement with MAYERSBACH (1956) staining according to PISCHINGER (1926) has been found excellent both in its simplicity and in the way in which the staining intensity gives a reflection of existing RNA concentrations. Aggregates of stained substance (Nissl substance) could be seen against a diffuse background of a lighter shade. This appearance was seen on the control side as well as on the operated side during all stages of regeneration. There was never any histological evidence of a diminished staining with methylene blue nor any signs of dissolution of the Nissl substance.

In contrast with the 7 μ sections those cut at 70 μ were particularly informative about volume change. Even after 4 days a definite increase in cell size was found, while after 20–50 days the cells were at their largest. The cells became equal in size on both sides again after 151 days.

According to SILVER (1942) the motoneurons of the lateral column innervate limb musculature in the frog. In agreement with this it was found that there was a massive reaction of the cell bodies in this column in its distal parts after sciatic nerve crush.

VOL. 5 1959/6

RNA and volume measurements. An effect on RNA content and cell volume was noted as early as 4 days when the first determinations were made. In both cases an increase was found and this change continued until 30–40 days, which roughly corresponds to the period of outgrowth of axons at the temperature in question (LUBINSKA and OLEKIEWICZ, 1950). It was further found that RNA and volume showed a parallel increase not only at this stage but during the whole cycle. Consequently a change in RNA concentration never occurred. After 30–40 days, when RNA content and volume had more than doubled, there was a slow return towards normal values during the following 3–4 months. This evidently corresponds to the time between the first establishment of peripheral contact and the final restitution to

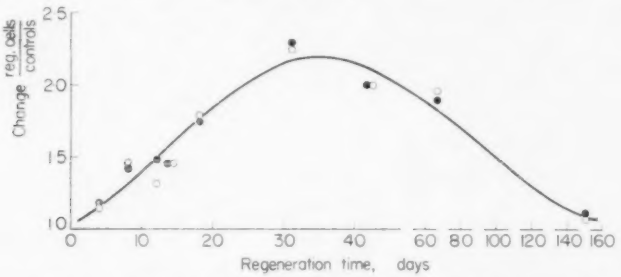


FIG. 1.—Changes in volume and RNA content in cell bodies of regenerating motoneurons of the frog. Filled circles RNA content, open circles cell body volume.

normal conditions, i.e. the period of maturation of nerve fibres (GUTMANN and SANDERS, 1943), since this is the period during which the first weak responses to stimulation gradually return to normal strength. The changes found are illustrated in Fig. 1. Table 1 shows the results of the analyses.

Nucleolar hypertrophy was noted during the whole process. Preliminary determinations of the RNA content in isolated nucleoli showed that the amount of RNA also increased during the whole cycle (Table 2).

TABLE 2.—RIBONUCLEIC ACID (RNA) AND VOLUME CHANGES IN NUCLEOLI OF SPINAL MOTONEURONS OF THE FROG DURING AXON REGENERATION. (THE RNA VALUES GIVE THE MEAN OF 5 DETERMINATIONS, THE DIAMETER VALUES ARE THE MEAN OF 30 MEASUREMENTS)

Regen. time (days)	RNA content ($\mu\mu$g) per nucleolus			Nucleolar diameter (μ)	
	Control cells	Regen. cells	Regen. / Controls	Control cells	Regen. cells
12	4·4	9·2	2·1	5·90	6·55
31	4·5	10·1	2·2	5·35	6·65
42	3·1	6·7	2·2	5·35	5·78

DISCUSSION

Comparing the present results with those of BRATTGÅRD *et al.* (1957) on axon regeneration in hypoglossal neurons of the rabbit there are certain similarities as

TABLE 1.—RIBONUCLEIC ACID (RNA) AND VOLUME CHANGES IN CELL BODIES OF SPINAL MOTONEURONS OF THE FROG DURING AXON REGENERATION. MEAN VALUES ± S.E.M.

Regen. time. (days)	RNA content ($\mu\mu$g) per cell body				Cell body volume (10^3 μ^3)				RNA concentrations (%, w/v)		
	Control cells	Regen. cells	Regen. / Controls	P	Control cells	Regen. cells	Regen. / Controls	P	Control cells	Regen. cells	Regen. / Controls
4	418 ± 20	489 ± 7	1·17	0·02	16·7 ± 1·5	19·0 ± 1·0	1·14	—	2·59	2·59	1·00
8	290 ± 21	411 ± 13	1·42	0·001	11·2 ± 1·5	16·3 ± 1·5	1·46	0·05	2·66	2·59	0·97
12	405 ± 9	595 ± 27	1·47	0·001	19·0 ± 1·1	24·9 ± 2·5	1·31	—	2·16	2·46	1·14
14	393 ± 30	573 ± 51	1·46	0·02	15·1 ± 1·0	22·0 ± 1·3	1·46	0·02	2·62	2·61	1·00
18	446 ± 36	774 ± 46	1·74	0·01	13·6 ± 1·1	24·2 ± 2·5	1·78	0·01	3·31	3·21	0·97
31	265 ± 20	607 ± 54	2·29	0·001	14·6 ± 1·2	33·0 ± 1·5	2·26	0·001	1·88	1·83	0·98
42	286 ± 12	577 ± 35	2·02	0·001	13·7 ± 0·6	27·8 ± 2·2	2·03	0·001	2·10	2·10	1·00
66	379 ± 36	730 ± 62	1·93	0·01	11·0 ± 1·1	21·6 ± 2·4	1·97	0·01	3·50	3·43	0·98
151	274 ± 19	292 ± 23	1·07	—	12·0 ± 0·7	13·1 ± 1·2	1·10	—	2·30	2·29	1·00

VOL. 5
1959/6

well as differences. Thus the RNA content of the cell bodies in both cases increases to about the same extent (slightly more than twice). One difference is that in the frog the increase starts immediately, while in the rabbit there is a period of about 10 days with unchanged values. A curious fact is that the rate of relative RNA increase is roughly the same in the two species in spite of the difference in body temperature and metabolism. This similarity contrasts with the well-known temperature sensitivity of the regeneration rate of peripheral nerves (LUBINSKA and OLEKIEWICZ, 1950; GAMBLE, 1957). As similar changes in volume and RNA content occur during the cycle in the frog, there is never any change in RNA concentration. Quite other

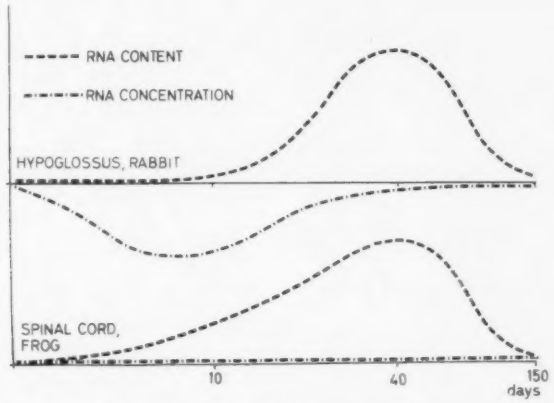


FIG. 2.—Diagram of changes in RNA content and concentration in cell bodies of regenerating motoneurons.

conditions are found in the hypoglossal neurons of the rabbit. Here, owing to a swelling of the cell body at a time when the amount of RNA has not altered, there is a considerable decrease in concentration. Fig. 2 shows the main changes in content and concentration in the two cases. The results of GERSH and BODIAN (1943) on the spinal cord of the rhesus monkey indicate that the conditions here are the same as for the rabbit hypoglossus. They found a decrease in RNA concentration and increased cell volumes. Thus the difference in reaction of rabbit hypoglossal and frog spinal cord cells is probably not due to a difference in their location in the nervous system.

The finding that the distribution and concentration of RNA need not necessarily be changed during axon regeneration may explain why chromatolysis has not always been recorded in this process (BUCY, 1928; GEIST, 1933). Thus it seems that the reaction of the cell body to axon severance is variable and possibly it need not react at all. Nor would there be any absolute need for it, as according to WEISS and HISCOE (1948), SAMUELS, BOYARSKY, GERARD, LIBET and BRUST (1951) and OCHS and BURGER (1958) there is already normally a continuous centrifugal flow in the axon. Cell hypertrophy then would only serve the purpose of hastening growth. For the frog at least it does not seem likely that the hypertrophy is due to a temporary arrest in the normal centrifugal transport of axoplasm. On the contrary, there is reason to believe that it is caused by increased synthesis in the cell body. Thus axoplasm is known to have a much lower RNA concentration than cytoplasm, 0·1 per cent as

compared to 1·7 per cent (NURNBERGER, ENGSTRÖM and LINDSTRÖM, 1952), a circumstance which would have been noted as a decrease in RNA concentration in the cell bodies. Furthermore, the nucleolus was found to increase in volume and RNA content, like the cell bodies, which it would have no reason to do were the hypertrophy due to the damming of the axoplasm.

The cause of the generally held view that RNA is lost from cell bodies of motoneurons during nerve regeneration may be partly explained by the confusion between changes in concentration and changes in amount. Possibly some of the results could be explained by the occurrence of degenerating cells (GOERING, 1928; TURNER, 1943). The uneven distribution of RNA in nerve cells is another factor which makes its evaluation from histological sections difficult. A similar discrepancy between the results of histophotometry and microchemistry was found during neurosecretion (EDSTRÖM and EICHNER, 1958).

As a general hypertrophy occurs, probably many cellular constituents show an increase. Thus the present findings do not warrant any conclusions about a specific role of RNA during the synthesis of the axoplasm necessary for nerve regeneration. They fit, however, with what is known from other studies about the relation between the rate of protein synthesis and the cellular content of RNA (see BRACHET, 1957, for ref.).

VOL.
5
1959/6

SUMMARY

The ribonucleic acid (RNA) content and the volume of individual cell bodies of motoneurons of the frog *Rana temporaria* were measured during chromatolysis after sciatic nerve crush. Preliminary determinations of nucleolar volume and RNA content were also performed. The following results were obtained:

(1) There was a parallel increase in volume and RNA as early as the fourth day onwards. Maximal values (about 250 per cent of the control side) were reached after about 5 weeks. After this time there was a slow regression in these values, which became normal again after 4–5 months.

(2) There was never any change in RNA concentration in the cell bodies in contrast to chromatolysed hypoglossal neurons.

(3) The results indicate that the changes represent a true hypertrophy and are not caused by a temporary arrest in transport of axoplasm. Increased nucleolar volumes and RNA values suggest that the hypertrophy is due to an increased synthesis.

Acknowledgements—Dr. P. M. B. WALKER, University of Edinburgh, kindly revised the English text of this paper. Mrs. MONA PERSSON skilfully assisted throughout the work.

REFERENCES

BRACHET J. (1957) *Biochemical Cytology*. Academic Press, New York.
BRATTGÅRD S. O., EDSTRÖM J.-E. and HYDÉN H. (1957) *J. Neurochem.* **1**, 316.
BUCY P. C. (1928) *J. comp. Neurol.* **45**, 129.
EDSTRÖM J.-E. (1958) *J. Neurochem.* **3**, 100.
EDSTRÖM J.-E. and EICHNER D. (1958) *Z. Zellforsch.* **48**, 187.
GAMBLE H. J. (1957) *Nature Lond.* **180**, 146.
GEIST F. D. (1933) *Arch. Neurol. Psychiat.* **29**, 88.
GERSH I. and BODIAN D. (1943) *J. cell. comp. Physiol.* **21**, 253.
GOERING J. H. (1928) *J. comp. Neurol.* **46**, 125.
GUTMANN E. and SANDERS F. K. (1943) *J. Physiol.* **101**, 489.
HYDÉN H. (1943) *Acta physiol. scand.* Suppl. 17.

LUBINSKA L. and OLEKIEWICZ M. (1950) *Acta Biol. exp., Varsovie* **15,** 125.
MAYERSBACH H. (1956) *Acta Histochem.* **3,** 128.
MICKLEWRIGHT H. L., KURNICK N. B. and HODES R. (1953) *Exp. Cell Res.* **4,** 151.
NISSL F. (1892) *Allg. Z. Psychiat.* **48,** 197.
NURNBERGER J., ENGSTRÖM A. and LINDSTRÖM B. (1952) *J. cell. comp. Physiol.* **39,** 215.
OCHS S. and BURGER E. (1958) *Amer. J. Physiol.* **194,** 499.
PISCHINGER A. (1926) *Z. Zellforsch.* **3,** 169.
SAMUELS A. J., BOYARSKY L. L., GERARD R. W., LIBET B. and BRUST M. (1951) *Amer. J. Physiol.* **164,** 1.
SILVER M. L. (1942) *J. comp. Neurol.* **77,** 1.
TURNER R. S. (1943) *J. comp. Neurol.* **79,** 73.
WEISS P. and HISCOE H. (1948) *J. exp. Zool.* **107,** 315.

OL.
5
9/60

Journal of Neurochemistry, 1959, Vol. 5, pp. 50 to 61. Pergamon Press Ltd. Printed in Northern Ireland

ALTERNATE PATHWAYS OF PYRIDINE NUCLEOTIDE OXIDATION IN CEREBRAL TISSUE*

A. GIUDITTA† and H. J. STRECKER‡
Department of Biochemistry, Albert Einstein College of Medicine,
Yeshiva University, New York, N.Y.

(*Received* 25 *February* 1959)

VOL.
5
1959/6

THE oxidation of reduced pyridine nucleotides by mammalian tissue has been studied with preparations chiefly derived from heart and skeletal muscle, kidney, and liver (for reviews see CHANCE, 1954; VELICK, 1956; MAHLER, 1956). These studies have provided evidence for two alternate pathways of DPNH and TPNH oxidation. One, principally responsible for the coupling of metabolic oxidations to the esterification of inorganic phosphate, appears to be associated with mitochondrial particles and is strongly inhibited by antimycin A and amylobarbitone (Amytal). The other pathway, whose function is as yet unknown, appears to be associated with microsomal particles and is not inhibited by either antimycin A or amylobarbitone. Some investigators have suggested that in addition liver mitochondria contain an external oxidative pathway for DPNH which is insensitive to antimycin A and amylobarbitone and is non-phosphorylating. The possible interrelationships among these systems have received both experimental and theoretical attention (LEHNINGER, 1951, 1954; ERNSTER, JALLING, LÖW and LINDBERG, 1955; DE DUVE, PRESSMAN, GIANETTO, WATTIAUX and APPELMANS, 1955; ERNSTER and LINDBERG, 1958).

Prior to the present time, studies of these electron transport mechanisms have not been pursued extensively with tissues of the nervous system. BRODY, WANG and BAIN (1952) have studied the distribution of DPNH-cytochrome *c* reductase and of cytochrome oxidase in four subcellular fractions separated from rat cerebral cortex by a modification of the SCHNEIDER and HOGEBOOM (1950) method of differential centrifugation developed for liver. The cytological nature of their preparations was not completely determined. It seemed especially interesting to study in cerebral tissue alternative pathways of pyridine nucleotide oxidation, since cerebral tissue function is so strikingly dependent on pyridine nucleotide linked glucose metabolism source of energy (MCILWAIN, 1955).

We are reporting here some of the results of our studies in which we have attempted to determine the existence of these alternate pathways in nervous tissue, as a their subcellular distribution, and some of their biochemical characteristics.

MATERIALS AND METHODS

Chemical preparations. DPNH, TPNH, and cytochrome *c* (horse heart) were obtained from the Sigma Chemical Co. Antimycin A, obtained from the Wisconsin Alumni Research Foundation, was

* Aided by grant No. B-1422 from the National Institutes of Health.
† Fellow of the National Multiple Sclerosis Society.
‡ Senior Research Fellowship (SF-42) of the Public Health Service.

made up to final concentration in 42·5% (v/v) alcohol. Amylobarbitone (Amytal, sodium 5-ethyl-5-isoamylbarbiturate) was a gift from Gane and Ingram, Inc. 5′-AMP was obtained from Pabst Laboratories, and β-napthyl acetate from the Mann Research Laboratories.

Assay procedures. All spectrophotometric assays were conducted at a temperature maintained between 28° and 30° in the Beckman DU spectrophotometer with cells of 1 cm light path.

DPNH oxidase. The rate of oxidation of DPNH was measured by following the change in optical density of 340 mμ, readings being taken at 0·5 or one min intervals. Preincubation of the cell fractions in water or EDTA, which has been reported to stimulate the activity of liver fractions (DE DUVE *et al.*, 1955), had no effect upon our preparations. However, the high recovery of DPNH oxidase activity (Table 2) might suggest some release of 'latent' activity brought about by the rehomogenization and centrifugation procedures involved in the separation of the cell fractions. The concentration of reduced pyridine nucleotides was determined by using the extinction coefficient, $E = 6{\cdot}22 \times 10^6/\text{cm}^2/\text{mol}$ (HORECKER and KORNBERG, 1948). The concentration of cytochrome *c* used for the experiments was determined from the optical density of the reduced and oxidized forms at 550 mμ, as obtained with excess sodium hydrosulphite and potassium ferricyanide respectively, and by use of a millimolar extinction coefficient of $27{\cdot}7 \times 10^3$ for reduced cytochrome *c* and $9{\cdot}2 \times 10^3$ for oxidized cytochrome *c* (MARGOLIASH, 1954).

The oxidation of DPNH was measured in a medium containing 0·3 μmole of DPNH and 100 μmoles of potassium phosphate buffer at pH 7·3 in a total volume of 3·0 ml. In the experiments with cytochrome *c*, this component was present in a concentration of 0·07 μmole/ml.

TPNH-cytochrome c reductase. The rate of reduction of cytochrome *c* was measured by following the change in optical density at 550 mμ, readings being taken at 30 sec intervals. Preincubation was also unnecessary for this enzyme system. The incubation medium contained 0·2 μmole of cytochrome *c*, 100 μmoles of potassium phosphate buffer, pH 7·3, 1 μmole of KCN, and 0·3 μmole of TPNH. With both the DPNH oxidase and TPNH-cytochrome *c* reductase determinations, the reactions were initiated by the addition of the tissue preparations, substrates were in excess, and under the conditions used linearity with regard to time and enzyme concentration was obtained.

Succinic-cytochrome c reductase. The medium and assay conditions were similar to those used for TPNH–cytochrome *c* reductase. However, in order to obtain linear rates it was found necessary to preincubate the cell fractions at 28°–30° for 7–9 min in the incubation medium and initiate the reaction by successive additions of KCN (1 μmole) and succinate (60 μmoles).

Cytochrome oxidase was measured by the method of STRAUS (1956). *Esterase* activity was determined from the rate of hydrolysis of β-napthyl acetate, by spectrophotometric observation at 328 mμ using a molar extinction coefficient of $1{\cdot}63 \times 10^6$ for β-napthol at this wave length. The assay medium contained 1·06 μmoles of β-napthyl acetate and 54 μmoles veronal buffer, pH 7·4, in a total volume of 3·0 ml. 5′-AMPase activity was determined according to HEPPEL and HILMOE (1951) with a substrate concentration of 8 μmoles/ml of 5′-AMP. Inorganic phosphate determinations were made according to the method of GOMORI (1941) on aliquots of the reaction mixture after 15 or 30 min of incubation at 37°. Total nitrogen was determined by microkjeldahl analysis.

Enzyme preparations. Rats of either sex, ranging in weight from 150 to 250 g, were used. The animals were decapitated, the brains removed in the cold, and the cerebrum was separated from the cerebellum by transverse section. Slices were removed from the cortex with a sharp blade, the amount of white matter being kept to a minimum. Slices from several animals were pooled and washed with 0·25 M-sucrose. After blotting dry with filter paper, the tissue was weighed and homogenized with 0·88 M-sucrose using the all glass homogenizer described by DOUNCE, WITTER, MONTY, PATE and COTTONE (1955). In general, not more than 4–5 excursions with each of the two pestles (loose and tight fitting) were necessary to obtain adequate dispersion. The suspension was diluted with 0·88 M-sucrose to a final concentration of 1 : 10 (w/v) and centrifuged according to the schedules shown in Table 1. The first three fractions were collected by means of the 4 place head of the multispeed attachment of the International refrigerated centrifuge, and RCF was calculated from the average radius. The remainder of the fractions were collected with the Spinco preparative centrifuge Model L, rotor no. 40, and RCF was calculated from the average radius. This technique is comparable to that used by NOVIKOFF, PODBER, RYAN and NOE (1953) for liver. Centrifugation time was measured after the desired rotational velocity had been reached. Supernatant suspensions were separated from the precipitates with capillary pipettes and as much as possible of the fluffy layers was left with the respective precipitates. The fractions were not washed and were suspended in a minimal volume of

TABLE 1.—CENTRIFUGAL FORCES USED TO SEPARATE FRACTIONS OF HOMOGENIZED RAT CEREBRAL CORTEX

Centrifugal force calculated according to the equation $RCF = 1118\, r\, N^2 \times 10^{-8}$

Fraction	Rotational speed (rev/min)	Centrifugal force g	Time (min)
1	890	1000	10
2	1500	3200	10
3	2600	9500	10
4	20,000	26,360	10
5	32,000	69,590	10
6	40,000	105,400	10
7	40,000	105,400	120

0·25 M-sucrose, the loose fitting pestle of the homogenizer being used to disperse the sediment. For those experiments in which fragmented particles were used, the dispersed particulate fraction was subjected to sonic vibration with a Raytheon sonic oscillator (Model S 102 A) for 5 min. The suspension was then centrifuged at 19,000 g for 15 min, and the sediment discarded.

The extent of variability in the experimental results can be estimated from the data of Table 2. Column 3 of this table presents the mean and the range of specific activities of the enzymes assayed in the total homogenate. Column 4 presents the recovery of the activities of all the fractions as a percentage of the activity present in the original homogenate.

RESULTS

Each of the seven fractions of subcellular particles was tested for DPNH oxidizing activity with and without addition of amylobarbitone (3×10^{-3} M) and antimycin A (3 μg/ml). These two compounds were inhibitory with all fractions, and the degree of inhibition was about the same for both compounds. The trace activity in the supernatant liquid of fraction 7 was neglected. The activity found in the presence of amylobarbitone or anti-mycin A is referred to as the insensitive activity; subtracting the values for insensitive activity from the total activity measurable in each fraction results in values which are referred to as the sensitive activity. The distributions of these two kinds of activities are shown in Fig. 1. In this and in the following figures, the relative specific activity (percentage of total activity/percentage of total nitrogen) of the enzymes of

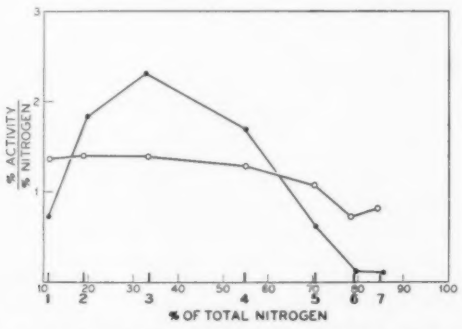


FIG. 1.—Distributions of DPNH oxidizing activity in fractions of rat cerebral cortex according to antimycin A and amylobarbitone sensitivity. Sensitive (●), insensitive (○). Conditions of assay described in test; numbers 1–7 refer to fractions.

VOL. 5 1959/6

TABLE 2.—ACTIVITIES AND RECOVERIES OF ENZYMES ASSAYED
Conditions are described in the text. The mean values are given with maximal and minimal activities in parentheses

Enzyme or enzyme system	No. of experiments	Specific activity μmoles substrate/min/mg N	Recovery %
Sensitive pathway of DPNH oxidation; cyt. *c* added to the reaction medium	4	0·096(0·090–0·106)	124(108–136)
Succinate-cyt. *c* reductase	5	0·042(0·025–0·076)	95(80–109)
Cytochrome *c* oxidase	3	5·75(4·12–7·38)*	89·3(85–93)
Insensitive pathway of DPNH oxidation; cyt. *c* added to the reaction medium	4	0·116(0·107–0·125)	88·5(84–93)
TPNH-cyt. *c* reductase	3	0·0095(0·0088–0·012)	88·6(84–91)
Esterase	3	0·350(0·344–0·356)	84·4(83–85)
5′-AMPase	3	0·0065(0·0055–0·0075)	88·9(85–94)

* Expressed as 'Quinone diimonium red' units

each fraction is plotted against the percentage of total nitrogen. The nitrogen content of each successive fraction is added to the sum of the preceding fractions, so that the total nitrogen content of all seven fractions plus supernatant fluid equals 100 per cent. This procedure is according to DE DUVE *et al.* (1955), and permits a ready comparison of fractions from different preparations with different nitrogen contents.

Sensitive activity

Since the sensitive DPNH oxidizing activity has been considered to be associated with mitochondria in liver (LEHNINGER, 1951; ERNSTER *et al.*, 1955; DE DUVE *et al.*, 1955) all of the brain fractions were also assayed for succinic-cytochrome *c* reductase and cytochrome oxidase, both of which are generally accepted as being characteristic mitochondrial enzymes in liver (cf. SCHNEIDER and HOGEBOOM, 1956). The distribution of these two enzymes in brain paralleled the sensitive DPNH-oxidase activity (Fig. 2). Although the parallelism is not exact, it is probable that the slight differences

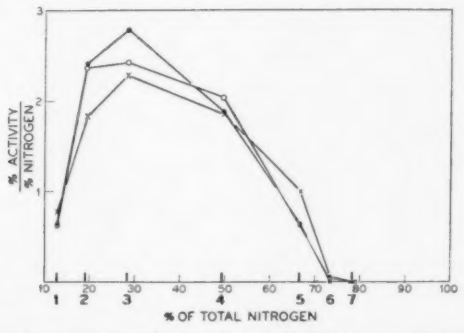


FIG. 2.—Distributions of antimycin A and amylobarbitone sensitive DPNH oxidizing activity (●), succinic-cytochrome *c* reductase (○) and cytochrome oxidase (×). Conditions described in text; numbers 1–7 refer to fractions.

represent the experimental variations of the methods* (Table 2). It should be noted at this point that, although the previous studies with both brain and liver fractions have measured DPNH-cytochrome *c* reductase activity, our own studies have been of DPNH oxidase. In all seven fractions the rate of oxidation of DPNH was never less than the rate of reduction of cytochrome *c*. However, there did seem to be some differences in the activities of these two enzyme systems which are now under investigation.

TPNH-cytochrome *c* reductase in liver fractions has been reported to be also sensitive to antimycin A (DE DUVE *et al.*, 1955). The low activity of TPNH oxidase activity in brain (less than 5% of DPNH oxidase) made it necessary to use the more sensitive reductase assay in our experiments.

Under our experimental conditions, there was little or no demonstrable antimycin A or amylobarbitone sensitive activity of TPNH-cytochrome *c* reductase. This is in sharp contrast to liver where 68 per cent of the total TPNH-cytochrome *c* reductase activity was reported to be sensitive (DE DUVE *et al.*, 1955).

From the foregoing observations it was concluded that the sensitive DPNH oxidase, succinic-cytochrome *c* reductase, and cytochrome oxidase, were associated with the same particulate fraction, but that this fraction did not contain a sensitive TPNH-cytochrome *c* reductase. In liver a similar particulate fraction has been identified as mitochondria. By analogy the same should be true for brain, and other investigators have so concluded (BRODY *et al.*, 1952). We have made electron microscope studies of these brain fractions and indeed the fraction containing the highest specific activity of these enzymes is richest in mitochondria, although other structures are also present (PETRUSHKA and GIUDITTA, 1959).

Insensitive activity

As demonstrated in Fig. 1, the distribution pattern of the insensitive DPNH oxidase was strikingly different from the sensitive one. The TPNH-cytochrome *c* reductase activity, which was completely insensitive to antimycin A and amylobarbitone, showed a somewhat similar pattern of distribution (Fig. 3). The shapes of these curves were reminiscent of the distribution curves of esterase,5′-AMPase and ribosenucleic acid found with liver fractions by NOVIKOFF *et al.* (1953), which components were attributed by these authors to microsomes. Since up to now similar studies with brain fractions have not been made, and no microsomal "markers" have been established for brain, it was of interest to examine the activity of these components in our fractions. Fig. 3 demonstrates the distribution pattern found for the four enzymes mentioned. The parallelism between esterase and 5′-AMPase is especially striking. Except for fraction 7 the specific activities for these two enzymes were parallel in all fractions. Even this difference may be more apparent than real, since linearity of enzyme activity with concentration was difficult to obtain for 5′-AMPase, and this was especially true for fraction 7, where competing reactions have considerable effect on the net inorganic phosphate released from 5′-AMP (unpublished).

The distributions of the insensitive DPNH oxidase activity and TPNH-cytochrome *c* reductase, on the other hand, differed from each other as well as from the esterase

* Fig. 1 represents a comparison of activities in the same preparations. Figs. 2 and 3 compare average activities obtained from several preparations.

VOL. 5 1959/6

and 5′-AMPase. These differences are especially striking in fractions 6 and 7. Preliminary determinations of ribosenucleic acid concentrations which have been previously reported (GIUDITTA and STRECKER, 1958) indicated that the distribution of this component paralleled the insensitive oxidizing activities only up to fraction 4. Ribosenucleic acid was especially concentrated in fraction 7 and in the supernatant fraction. Electron microscope studies of fraction 7 showed very few mitochondria, many membranous fragments, some granules, and other undefined structures (PETRUSHKA and GIUDITTA, 1959).

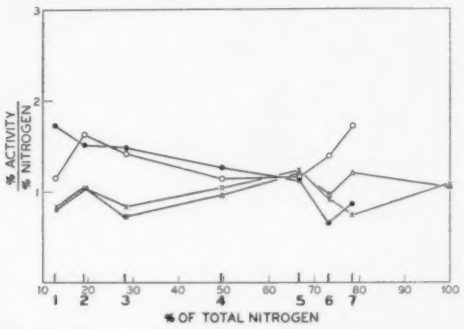


FIG. 3. Distributions of antimycin A and amylobarbitone insensitive DPNH oxidizing activity (●), TPNH-cytochrome *c* reductase (○), esterase (△), and 5′-AMPase (×). Conditions described in text; numbers 1–7 refer to fractions.

Consideration of the preceding data leads to the tentative conclusions that the insensitive DPNH oxidase and the TPNH-cytochrome *c* reductase of brain are not present on the same particles as the sensitive DPNH oxidase; that these particles containing the insensitive pathways are microsomal, possibly derived from the 'endoplasmic reticulum', and that, under our experimental conditions, fragments of 'endoplasmic reticulum' are present in all fractions which are relatively freer of RNA granules than the corresponding liver fractions. Our distribution patterns also suggest that these fragments, which may be equivalent to liver microsomes, are biochemically heterogeneous, and that perhaps the insensitive DPNH oxidase and the TPNH-cytochrome *c* reductase activities are associated with different particles than are esterase and 5′-AMPase.

Biochemical characterization of the two pathways of DPNH oxidation

In order to compare the biochemical properties of the sensitive and insensitive pathways, studies were initiated to determine the effects on these pathways of 3 factors: (a) addition of cytochrome *c*, (b) fragmentation, and (c) addition of ions.

Addition of cytochrome c. As previously mentioned, the oxidation of DPNH by all fractions was measured from the change in optical density at 340 mμ. However, it was observed that very little oxidation of DPNH could be obtained in the more slowly sedimenting fractions unless cytochrome *c* was added. Table 3 compares the ratios of activities (mean) of DPNH oxidation, with and without cytochrome *c* (added in a concentration sufficient to obtain maximal rates) in the homogenate, 'mitochondria' (fractions 3 and 4), and 'microsomes' (fractions 6 and 7). Stimulation of the sensitive pathway in all fractions was about two-fold. The stimulation of the insensitive pathway in fractions 6 and 7, which are relatively free of mitochondria,

is very great. In some experiments no oxidation of DPNH was obtained unless cytochrome *c* was added. These results appear to be consistent with the view that the insensitive pathway is entirely dependent upon added cytochrome *c* for oxidation of DPNH, and is probably similar to the microsomal DPNH-cytochrome *c* reductase which has been studied and purified from liver preparations (STRITTMATTER and BALL, 1952; STRITTMATTER and VELICK, 1956). The fact that the insensitive pathway in the mitochondrial fractions and in the homogenate is stimulated to a lesser extent by added cytochrome *c* is probably dependent either on the presence of free cytochrome *c* in these fractions or on the interaction with cytochromes present in other particles.

TABLE 3.—EFFECT OF ADDED CYTOCHROME *c* ON THE SENSITIVE AND INSENSITIVE PATHWAYS OF DPNH OXIDATION

Preparation	+Cytochrome *c* / −Cytochrome *c*	
	Sensitive	Insensitive
Homogenate (13 experiments)	2·35	16·7
Mitochondrial fraction (3 experiments)	2·16	11·3
Microsomal fraction (5 experiments)	2·02	99·0

Even with fraction 7, it was still possible to assay DPNH oxidation at 340 mμ when cytochrome *c* was added. Although the cytochrome oxidase activity of this fraction was only a small proportion of the total activity as measured by the oxidation of *p*-phenylenediamine (Fig. 3), the reoxidation of cytochrome *c* was not rate limiting.

Fragmentation. Fragmentation of the mitochondria-containing fractions by sonic vibration resulted in a preparation with somewhat different properties. The most striking change was a large increase in the DPNH oxidase activity. Under the conditions of our assay, this increase of activity was approximately five to six fold over the total DPNH oxidase activity (sensitive and insensitive combined) initially present in the unfragmented particles. Analysis of the activity of these fragmented particles in terms of amylobarbitone and antimycin sensitivity revealed that the increase had actually taken place only in the sensitive pathway, which had increased ten to twelve fold in activity, whereas the insensitive pathway either did not increase or actually decreased in activity (Table 4). As a consequence, whereas the activity of the intact mitochondria-containing fraction was 40–50 per cent inhibited by amylobarbitone or antimycin A, the activity of the sonically fragmented particles was about 90 per cent inhibited by these agents. In the experiment shown in Table 4, a fraction obtained from a 0·25 M-sucrose homogenate was used. Qualitatively similar results were obtained with 0·88 M-sucrose preparations but the increase in activity following fragmentation

VOL. 5 1959/6

was not as great. The differences between 0·25 M-sucrose and 0·88 M-sucrose homogenates are being further investigated.

Effect of ions. Further examination of the increased activity resulting from fragmentation revealed that a considerable portion of the increase depended on the buffer system used in the assay medium. Comparison of the activity of whole mitochondria and fragments therefrom in the absence of added buffer, but at the same pH, indicated a relatively small increase of activity on fragmentation. Addition of phosphate markedly stimulated the activity of the fragmented particles but slightly inhibited the activity of the unfragmented mitochondrial preparation (Fig. 4).

TABLE 4.—EFFECT OF SONIC OSCILLATION ON THE SENSITIVE AND INSENSITIVE PATHWAYS OF DPNH OXIDATION

Cytochrome *c* added to the reaction medium

Preparation	μmoles DPNH/min/mg N	
	Sensitive	Insensitive
Mitochondrial fraction	0·070	0·158
Mitochondrial fraction—subjected to sonic oscillation for 5 min	0·888	0·111

Phosphate was not unique in this regard; a number of other ions were also able to stimulate the activity of mitochondrial fragments. Table 5 compares the effectiveness of the compounds tested with the activity of the preparation without added ions. In the experiments where buffers were not used, the pH was adjusted to 7·1–7·3; cytochrome *c* was not added in any experiment. The pH optimum of this enzymic activity is quite broad and in this range there is no difference. The buffer systems used were also prepared in this range of pH. The comparative values shown are approximate; the activity without additions varied over a hundred per cent range for different preparations and the same was true for the activity measurements with

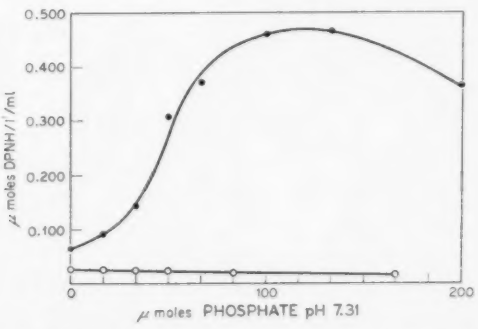


FIG. 4.—Effect of phosphate concentration on the sensitive pathway of DPNH oxidation; fragmented mitochondria (●), whole mitochondria (○). Homogenate prepared in 0·25 M-sucrose. Conditions described in text.

additions. The results presented in Table 5 appear to be somewhat similar to the stimulation of DPNH and succinate oxidation observed by various investigators with fragmented heart particles and not yet completely understood (KEILIN and HARTREE, 1949; SLATER, 1950; ALTMANN and CROOK, 1953; BONNER, 1954). It is not known whether or not this difference in behavior of the mitochondria and fragments therefrom is unique for brain tissue. Comparable studies with other tissues do not appear to have been carried out, although oxidation by intact heart sarcosomes has been reported not to be stimulated by histidine (SLATER and CLELAND, 1953). MCILWAIN and TRESIZE (1957) have reported that the oxidation of DPNH by brain homogenates made in

TABLE 5.—EFFECT OF IONS ON DPNH OXIDATION BY FRAGMENTED BRAIN MITOCHONDRIA

(Preparations similar to that used for experiments of Table 4 and Fig. 4)

Compound	Optimal concentration	Stimulation
Histidine	0·023 M	5–6 fold
Aspartate	0·023 M	5–6 fold
Pyrophosphate	0·033 M	8–9 fold
EDTA	0·050 M	7–8 fold
Borate	0·066 M	2–3 fold
Glycyl-glycine	0·066 M	2–3 fold
Tris(hydroxymethyl) -aminomethane	0·083 M	5–6 fold
Potassium chloride	0·083 M	3–4 fold
Sodium chloride	0·083 M	3–4 fold
Phosphate	0·10 M	7–10 fold

0·25 M-sucrose was stimulated by EDTA (ethylenediaminetetraacetic acid), phosphate, histidine, and NaCl. It is possible that the mitochondria in their preparation had already been sufficiently altered. The electron microphotographs of our fractions demonstrate that the mitochondria are relatively intact (PETRUSHKA and GIUDITTA, 1959). We are continuing our investigation of the mechanism of this phenomenon and the comparison of brain with other tissues.

DISCUSSION

The concept of external and internal mitochondrial pathways of oxidation of DPNH was originally proposed by LEHNINGER (1951, 1954) who characterized the external pathway as requiring exogenous cytochrome *c*, being non-phosphorylating, and not inhibited by antimycin A. LEHNINGER considered the possibility that the external pathway was due to microsomal contamination, but this possibility was contested by ERNSTER and LINDBERG (1958) who extended the characterization to include insensitivity to amylobarbitone. Chief support for an external pathway associated with mitochondria is obtained from the data of DE DUVE *et al.* (1955) which demonstrated that washed liver mitochondria had the same percentage ratio of antimycin A insensitive DPNH- and TPNH-cytochrome *c* reductases, whereas in microsomes the ratio of DPNH- to TPNH-cytochrome *c* reductase activities was much higher. These

VOL. 5 1959/6

investigators also found that their mitochondrial preparations were very low in glucose-6-phosphatase, an enzyme used as a marker for microsomes in liver. However, it cannot be ruled out at present that glucose-6-phosphatase and the antimycin A insensitive DPNH- and TPNH-cytochrome *c* reductase activities are associated with different microsomal particles which can be washed at different rates from the mitochondrial fractions. NOVIKOFF *et al.* (1953) have provided evidence for the biochemical heterogeneity of the microsomal fractions of liver, and our own data indicate that biochemical heterogeneity may also be present in the brain fractions. The practically complete absence of sensitive TPNH-cytochrome *c* reductase activity in rat brain preparations might mean that brain mitochondria differ from liver. However, it is also possible that the sensitive TPNH-cytochrome *c* reductase activity observed in liver consists of TPNH-DPN transhydrogenase coupled to DPNH-cytochrome *c* reductase and utilizing endogenous DPN. It has been reported that this transhydrogenase reaction does not occur in brain (KAPLAN, COLOWICK and NEUFELD, 1953). Removal of the TPNH-cytochrome *c* reductase system from liver mitochondria, and coupling of TPNH-DPN transhydrogenase with contaminating microsomal, insensitive DPNH-cytochrome *c* reductase as well as with sensitive, mitochondrial DPNH-cytochrome *c* reductase would account for the results of DE DUVE *et al.* (1955).

Some further insight into this problem may be gained by a comparison of heart, liver, and brain. BALTSCHEFFSKY and BALTSCHEFFSKY (1958) have recently reported that heart mitochondrial preparations had practically no insensitive DPNH oxidase activity. In brain the ratio of insensitive to sensitive DPNH oxidase activity varies from one to two (Tables 2 and 4). DE DUVE *et al.* (1955) have reported the insensitive activity in liver to be about three times the sensitive activity when reductase was assayed. Our own measurements of sensitive and insensitive activity in liver homogenates indicate a somewhat higher ratio which also agrees fairly well with the observations of BRODY *et al.* (1952) that the total DPNH-cytochrome *c* reductase activity is ten times higher in liver than in brain.

NOVIKOFF (personal communication) has also observed that in sections of heart stained for DPNH oxidizing activity only stained mitochondrial structures are seen; whereas in brain both mitochondria and the basophilic material (microsomes) were stained and in liver the basophilic material (microsomes) stained so intensely that the mitochondria were obscured. Thus the qualitative cytochemical data are consistent with the quantitative enzymic assays relating the level of insensitive activity in heart, liver, and brain to the DPNH oxidase activity in the microsomal fractions of the tissues.

The biochemical characteristics of these systems may now be considered. Our results with the brain preparations suggest that the amylobarbitone and antimycin A sensitive DPNH oxidase is firmly bound to the mitochondrial structure and that it is not markedly stimulated by cytochrome *c* as is the insensitive microsomal system. Fragmentation leads to a considerable enhancement of the sensitive pathway which is further stimulated by various ions. These effects are not observed for the insensitive pathway. It seems possible that liver mitochondria behave similarly since different effects have been reported on oxidative phosphorylation with liver mitochondria according to whether or not exogenous DPNH or endogenously generated DPNH is used as substrate. These effects seem to depend on the nature of the pretreatment of the preparations, the addition of cytochrome *c*, the incubation media used, etc.

(*cf.* LEHNINGER, 1951, 1954; ERNSTER *et al.*, 1955; MALEY, 1957). If it be accepted that the preparations used are contaminated with the microsomal, insensitive DPNH-cytochrome *c* reductase, then it seems reasonable that what has been observed reflects alterations in the relative activities of the two pathways brought about by the experimental conditions.

SUMMARY

The oxidation of reduced pyridine nucleotides by antimycin A and amylobarbitone sensitive and insensitive pathways has been studied with seven subcellular fractions obtained from rat cerebral cortex. The distribution of the sensitive pathway of DPNH oxidation parallels in general the distribution of enzymes which have been accepted as being located in mitochondria. The distribution of the insensitive pathway of DPNH and TPNH oxidation parallels the distribution of enzymes which are probably located in the endoplasmic reticulum. All of the TPNH-cytochrome *c* reductase in these preparations appears to be insensitive to antimycin A and amylobarbitone.

Preliminary biochemical studies on the two pathways indicate that the insensitive pathway is practically completely dependent on external cytochrome *c* for activity, and that fragmentation of mitochondria leads to considerable increase in activity of the sensitive pathway, which is markedly dependent on the ionic environment. It is proposed that these biochemical differences be used as further criteria for distinguishing the two pathways *in vitro*.

Acknowledgements—We gratefully acknowledge the contributions of Dr. SASHA ENGLARD, and the technical assistance of Mr. AMADEO ARBOLEDA.

REFERENCES

ALTMANN S. M. and CROOK E. M. (1953) *Nature, Lond.* **171,** 76.
BALTSCHEFFSKY H. and BALTSCHEFFSKY, M. (1958) *Biochim. biophys. acta* **29,** 220.
BONNER W. D. Jr. (1954) *Biochem. J.* **56,** 274.
BRODY T. M., WANG R. I. H. and BAIN J. A. (1952) *J. biol. Chem.* **198,** 821.
CHANCE B. (1954). In *The Mechanism of Enzyme Action*, (Edited by MCELROY W. D. and GLASS B.) p. 399. The Johns Hopkins Press, Baltimore.
DOUNCE A. L., WITTER R. F., MONTY K. J., PATE S. and COTTONE M. A. (1955) *J. biophys. biochem. Cyt.* **1,** 139.
DE DUVE C., PRESSMAN B. C., GIANETTO R., WATTIAUX R. and APPELMANS F. (1955) *Biochem. J.* **60,** 604.
ERNSTER L., JALLING O., LÖW H. and LINDBERG O. (1955) *Exp. Cell Res.* Suppl., **3,** 124.
ERNSTER L. and LINDBERG O. (1958) *Ann. Rev. Physiol*, **20,** 13.
GIUDITTA A. and STRECKER H. J. (1958) *IVth International Congress of Biochemistry* p. 72.
GOMORI G. (1941–42) *J. Lab. clin. Med.* **27,** 955.
HEPPEL L. A. and HILMOE R. J. (1951) *J. biol. Chem.* **188,** 665.
HORECKER B. L. and KORNBERG A. (1948) *J. biol. Chem.* **175,** 385.
KAPLAN N. O., COLOWICK S. P. and NEUFELD E. F. (1953) *J. biol. Chem.* **205,** 1.
KEILIN D. and HARTREE E. F. (1949) *Biochem. J.* **44,** 205.
LEHNINGER A. L. (1951) In *Phosphorus Metabolism* (Edited by MCELROY W. D. and GLASS B.) Vol. 1, p. 344. The Johns Hopkins Press, Baltimore.
LEHNINGER A. L. (1954) *The Harvey Lectures* **47,** 176.
MAHLER H. R. (1956) *Advanc. Enzymol.* **17,** 233.
MALEY G. F. (1957) *J. biol. Chem.* **224,** 1029.
MARGOLIASH E. (1954) *Biochem. J.* **56,** 535.
MCILWAIN H. (1955) In *Biochemistry and the Central Nervous System.* Little, Brown, Boston.
MCILWAIN H. and TRESIZE M. A. (1957) *Biochem. J.* **65,** 288.

VOL.
5
1959/6

NOVIKOFF A. B., PODBER E., RYAN J. and NOE E. (1953) *J. Histochem. Cytochem.* **1, 27.**
PETRUSHKA E. and GIUDITTA A. (1959) *J. biophys. biochem. Cytol.* **6,** 129.
SCHNEIDER W. C. and HOGEBOOM G. H. (1950) *J. biol. Chem.* **183,** 123.
SCHNEIDER W. C. and HOGEBOOM G. H. (1956) *Ann. Rev. Biochem.* **25, 201.**
SLATER E. C. (1950) *Biochem. J.* **46,** 484.
SLATER E. C. and CLELAND K. W. (1953) *Biochem. J.* **53,** 357.
STRAUS W. (1956) *Biochim. biophys. acta* **19,** 58.
STRITTMATTER C. F. and BALL E. G. (1952) *Proc. nat. acad. Sci. Wash.* **38, 19.**
STRITTMATTER P. and VELICK S. F. (1956) *J. biol. Chem.* **221,** 277.
VELICK S. F. (1956) *Ann. Rev. Biochem.* **25,** 257.

OL.
5
9/60

Journal of Neurochemistry, 1959, Vol. 5, pp. 62 to 67. Pergamon Press Ltd. Printed in Northern Ireland

THE SYNTHESIS OF UREA IN THE LIVING RAT BRAIN*

MICHAEL B. SPORN, WESLEY DINGMAN, ALFRED DEFALCO and ROBERT K. DAVIES†
Department of Psychiatry, University of Rochester
School of Medicine and Dentistry, Rochester, New York

(*Received* 28 *January* 1959)

THE problem of ammonia detoxication in brain continues to attract the attention and effort of many investigators, for there has not yet evolved a definitive and comprehensive picture of the manner in which the brain handles the potentially toxic substance ammonia. In an excellent review of the field, STRECKER (1957) has surveyed the data which suggest that the glutamic acid-glutamine system is the most likely candidate for ammonia removal in brain. He also notes that 'although free ornithine, citrulline, and arginine are present in brain (TALLAN, MOORE and STEIN, 1954), the enzyme systems participating in urea synthesis *via* the Krebs mechanism (KREBS and HENSELEIT, 1932) have not been described thus far in nervous tissue.' Recently, we have described a new method for studying metabolic pathways in the brain of the intact animal, and have shown that rat brain can synthesize ornithine and arginine from proline (SPORN, DINGMAN and DEFALCO, 1959). It was suggested that this pathway might supplement the glutamic acid–glutamine system in detoxifying the brain. The present experiments, which utilize the same method that enabled us to demonstrate that brain can metabolize proline to arginine, indicate that brain can also metabolize arginine to urea. It thus appears that the living brain is capable of urea synthesis.

METHODS

Injection of rats and preparation of brain homogenates. Four male albino rats, three months old, of the Sprague-Dawley strain (Holtzman Co., Madison, Wis.), were used in these experiments. All injections, both intracisternal and intravenous, were performed under light ether anaesthesia, by use of a microsyringe calibrated to 0·0001 ml (Nuclear Science Associates, 55 Manor Drive, Newark, N. J.). All injections, both inert and radioactive, consisted of 1·2 mg of L-arginine·HCl in a volume of 0·01 ml of water, the radioactive L-arginine·HCl having an activity of 37 μC. Rats 2 and 4 were injected intracisternally with 1·2 mg of uniformly labelled [^{14}C]-L-arginine·HCl; five minutes after their intracisternal injections these same rats were injected in the exposed femoral vein with 1·2 mg of inert L-arginine·HCl. Rats 1 and 3 (littermates, respectively, of rats 2 and 4) were injected intracisternally with 1·2 mg of inert L-arginine·HCl, and five minutes later were injected in the exposed femoral vein with 1·2 mg of uniformly labelled [^{14}C]-L-arginine·HCl. All rats were alert and walking in a coordinated manner within a few minutes after injections were finished.

The animals were sacrificed by decapitation one hr after the injection of radioactive arginine. Their brains were rapidly removed, cleaned of meninges, blotted with filter paper to remove any surface blood, weighed on a torsion balance, and homogenized in a Potter–Elvehjem homogenizer in 8 ml of 5% trichloroacetic acid (TCA).

Isolation of urea from brain. Inert carrier urea (Merck reagent), 8 mg in 2 ml of water, was added to each of the homogenates, followed by additional TCA to bring the final TCA concentration to 10%. Each homogenate was centrifuged, and the clear supernatant solution was decanted and saved. The precipitate was washed twice with 5 ml portions of 10% TCA, followed by centrifugation.

* Supported by grant M-1901 from the National Institute of Mental Health, United States Public Health Service and by the Foundations' Fund for Research in Psychiatry.

† Summer Research Fellow, Department of Biochemistry (supported by a U.S. P.H.S. Experimental Training Grant to the University of Rochester).

VOL.
5
1959/6

The supernatant solutions from all three centrifugations were pooled as the acid-soluble fraction.

The acid-soluble fraction was extracted three times in a separatory funnel with 25 ml portions of CCl_4, and the CCl_4 was discarded. Celite Analytical Filter Aid (8 ml of powder) was added, the solution centrifuged, and the supernatant saved. The Celite was washed twice with water and the washings were added to the above supernatant solution. The total acid-soluble fraction was taken down almost to dryness in a rotary vacuum evaporator, with care to keep the temperature below 50°. One ml of N-HCl was added and the resulting solution was put on a 50 cm × 4 cm^2 column of 200–400 mesh Dowex 50-X4, equilibrated with N-HCl. The column was eluted with N-HCl at a flow rate of 60 ml/hr, and 10 ml fractions were collected. In this system the urea peak occurs at approximately 600 ml of effluent. (It had previously been found that no less than 96% of an 8 mg load of urea may be recovered from such a column.) In order of elution, aspartic acid, serine, threonine, glutamic acid, glycine, and alanine all travel ahead of urea; γ-aminobutyric acid travels with urea; and valine, proline, leucine, isoleucine, ornithine, lysine, histidine, and arginine all travel significantly behind urea. The urea-containing portion of the effluent from the Dowex 50 column was taken to dryness on a rotary vacuum evaporator, with care to keep the temperature below 50°.

Precipitation and recrystallization of dixanthydryl urea. The dry residue from the above evaporation was dissolved in 5 ml of 80% acetic acid, and 250 mg of xanthydrol (Eastman Kodak), dissolved in 4 ml of glacial acetic acid, was added. A copious, finely divided, white precipitate of dixanthydryl urea formed within a minute, and the mixture was allowed to stand at room temperature for 1 hr, after which the precipitate was collected on Whatman No. 50 paper in a Büchner funnel, washed with glacial acetic acid and methanol, and dissolved in pyridine. Aliquots of this solution of dixanthydryl urea in pyridine were used for determination of radioactivity and colorimetric estimation of the amount of dixanthydryl urea present. The dixanthydryl urea was recrystallized from pyridine-water to constant specific activity; after each recrystallization it was washed with pyridine–water (1 : 1) and methanol, and then redissolved in pyridine for determination of specific activity.

Determination of radioactivity. All counting was done in the Geiger region with a gas flow counter fitted with an end-window. All samples were at infinite thinness and were plated in duplicate. Samples from rats 1 and 3 were counted for no less than 2000 counts, whereas samples from rats 2 and 4 were counted for no less than 20,000 counts. All data reported have been corrected for background radiation.

Chemical analyses. Urea was determined by Nesslerization of the ammonia liberated by the action of urease on samples buffered to pH 7·0. Any tendency toward turbidity during Nesslerization was controlled by addition of 0·05 ml of an aqueous solution containing 2% iodine and 3% KI to the Nesslerization mixture (CONNERTY, BRIGGS and EATON, 1955).

Arginine was determined by the method of ROSENBERG, ENNOR and MORRISON (1956).

Dixanthydryl urea was determined by the yellow colour that this compound gives with 50% (v/v) H_2SO_4 (ENGEL and ENGEL, 1947). Five ml of 50% H_2SO_4 were added to 0·10 ml aliquots of dixanthydryl urea in pyridine, and optical density at 390 mμ measured with a Beckman DU spectrophotometer.

Radioactive Arginine. [^{14}C]-L-arginine·HCl, uniformly labelled, (Nuclear-Chicago Corp., Chicago, Ill.) was recrystallized from acetone-isopropanol-water and washed with acetone prior to use. When chromatographed in both butanol-acetic acid-water (25 : 6 : 25) and pyridine–acetic acid–water (10 : 7 : 3), this material gave a single ninhydrin positive spot and a single peak of radioactivity, both of which corresponded to a known sample of L-arginine·HCl.

RESULTS

The total radioactivity in the acid-soluble fractions from rats 2 and 4 (which received the radioactive arginine intracisternally) was approximately 200 times greater than in the corresponding fractions from rats 1 and 3 (which received the radioactive arginine intravenously (Table 1)).

The dixanthydryl urea isolated from rats 2 and 4 was 35–37 times more radioactive than that isolated from their respective littermate controls, rats 1 and 3 (Table 2). Total radioactivity in brain urea was calculated by multiplying the final specific activity of the dixanthydryl urea (in counts per minute per mg) by (420/60) × (8·0 + 0·5), where 420 is the mol. wt. of dixanthydryl urea, 60 is the mol. wt. of

TABLE 1.—RADIOACTIVITY IN BRAIN ACID-SOLUBLE FRACTIONS OF RATS SACRIFICED ONE HOUR AFTER INJECTION OF RADIO-ARGININE

Rat	1	2	3	4
Injections*	Intracisternal inert-arginine *followed by* intravenous radio-arginine	Intracisternal radio-arginine *followed by* intravenous inert-arginine	Intracisternal inert-arginine *followed by* intravenous radio-arginine	Intracisternal radio-arginine *followed by* intravenous inert-arginine
Weight of animal (g)	350	340	330	360
Brain wet weight (g)	1·680	1·750	1·750	1·760
Total activity of injected arginine (counts/min)	27,000,000	27,000,000	27,000,000	27,000,000
Total activity of brain acid-soluble (counts/min)	29,000	8,100,000	42,000	8,100,000

* All arginine was injected as the monohydrochloride.

TABLE 2.—SPECIFIC ACTIVITY AND TOTAL ACTIVITY OF UREA (ISOLATED AS DIXANTHYDRYL UREA) FROM BRAIN

Rat		1	2	3	4
Injections*		Intracisternal inert-arginine *followed by* intravenous radio-arginine	Intracisternal radio-arginine *followed by* intravenous inert-arginine	Intracisternal inert-arginine *followed by* intravenous radio-arginine	Intracisternal radio-arginine *followed by* intravenous inert-arginine
Specific activity of dixanthydryl urea (counts/min/mg dixanthydryl urea)	Initial precipitation	43	1,570	52	1,590
	First recrystallization	44	1,610	39	1,690
	Second recrystallization	43	1,630	43	1,710
	Third recrystallization	43	1,500	46	1,710
Total radioactivity in brain urea (counts/min)		2,600	89,000	2,700	101,000

* All arginine was injected as the monohydrochloride.

VOL. 5 1959/60

urea, 8·0 is the number of mg of carrier urea added to each brain acid-soluble fraction, and 0·5 is the number of mg of urea normally found in a rat brain of weight 1·7–1·8 g. Since most of the radioactive material in the initial brain extract was separated from urea by the ion exchange procedure, and since the formation of dixanthydryl urea is a highly specific method for isolating urea, it is not unreasonable to expect to isolate a derivative which shows constant specific activity over several recrystallizations. The specificity of the reaction of urea with xanthydrol is shown by the fact that under the conditions used in our experiments none of the following compounds give a precipitate with xanthydrol: glutamine, asparagine, ureidosuccinic acid, arginine, guanidine, γ-guanidinobutyric acid, guanidinoacetic acid, creatine, creatinine, glycine, alanine, glutamic acid, γ-aminobutyric acid, valine, proline, ethanolamine, glucosamine, glucose, ribose, glucose-6-PO_4, glucose-1-PO_4, glyceraldehyde, lactic acid, oxaloacetic acid, guanine, adenine, and cytosine. The dixanthydryl urea isolated from the four rats gave a sharp melting point (with simultaneous decomposition) at 300°–301° and an identical mixed melting point with an authentic sample of dixanthydryl urea. Melting points were obtained with a Fisher–Johns melting point apparatus, calibrated with anthraquinone.

DISCUSSION

The data presented indicate that brain can form urea from arginine. The possibility that the radioactive urea found in brain was formed in organs other than brain has been controlled by the paired littermate injections; there is a strikingly greater amount of radioactivity in brain urea after an intracisternal injection of a dose of radioactive arginine than after intravenous injection of the identical dose of radio-arginine. Moreover, the possibility that an intracisternal injection of arginine damages the blood-brain barrier, so that urea formed in other organs from radio-arginine (which has leaked out of the brain) would then enter the brain quite readily, has been controlled by the intracisternal injections of inert arginine in rats 1 and 3.

It might be argued that all of the intravenously injected ^{14}C-arginine, in rats 1 and 3, was rapidly metabolized by the liver to urea and that this urea was rapidly excreted, resulting in low blood levels of radioactive urea at the time of sacrifice; whereas, in rats 2 and 4, the intracisternally injected ^{14}C-arginine entered the blood circulation over a more prolonged period of time, thus leading to blood levels of radioactive urea (produced by extra-cerebral tissues from this ^{14}C-arginine) which would be higher at the end of one hour than those from rats 1 and 3. With this line of reasoning, our results might reflect only the amount of radioactive urea found in the blood contaminating the brain at the time of sacrifice. However, the following simple calculations show that blood contamination cannot possibly account for our results. Each rat was injected with uniformly labelled ^{14}C-arginine with an activity of 27,000,000 counts/min. At the time of sacrifice, rats 2 and 4 each had approximately 8,000,000 non-urea counts/min isolated from the brain acid-soluble fraction. There are then approximately 19,000,000 counts/min of the original injection which are not accounted for. If one assumes that *all* of this radioactivity were converted to urea by extra-cerebral tissues, that *none* of it had been excreted, and that *all* of it were present in the blood at the time of sacrifice, then the maximal number of counts/min that could contaminate the brains of rats 2 and 4 would be approximately 15,000. This figure is based on the fact that only 0·08 per cent of the total blood volume is

present in the brain of an exsanguinated rat (SHARPE, CULBRETH and KLEIN, 1950).

Another potential source of error in our experiments would be the hydrolysis, during the process of extraction and isolation of brain urea, of the amidine group of arginine (or of some other compound with which arginine had transamidinated) to yield urea. However, it was found that arginine, guanidinoacetic acid, and γ-guanidinobutyric acid were all stable to acid hydrolysis under conditions equivalent to or stronger than those used in our experiments.

Inasmuch as rat brain has previously been shown to be capable of synthesizing arginine from proline (SPORN, DINGMAN and DEFALCO, 1959), and as there is now evidence that brain forms urea from arginine, it is reasonable to conclude that urea synthesis is feasible within the living brain.

In a classical set of experiments, BOLLMAN, MANN, and MAGATH (1924) demonstrated that 'the production of urea in the body of the dog is entirely dependent on the presence of the liver, since urea formation ceases completely as soon as the liver is removed.' In reviewing the problem of urea formation, BALDWIN (1957) notes that the observation that hepatectomy in the dog leads to cessation of urea production 'points to the liver as the sole seat of urea synthesis in the mammalian organism.' However, an alternative interpretation of such experiments with hepatectomized animals is possible, namely, that the functional integrity of the liver is necessary for normal metabolism in other organs, and such extra-hepatic metabolism may include urea synthesis. GEIGER *et al.* (1954, 1956) have shown that perfused brain needs factors elaborated or released by the liver to maintain normal metabolism; in the absence of such liver factors a perfused brain preparation rapidly deteriorates.

At present, there is little information about the intermediates involved in urea synthesis in brain, in contrast to the wealth of data obtained from liver preparations (KREBS and HENSELEIT, 1932; COHEN, 1954; RATNER, 1954; BACH and SMITH, 1956; BRONK and FISHER, 1956; MILLER *et al.*, 1956). Moreover, one cannot yet assess the quantitative significance of urea synthesis as a mode of ammonia detoxication in brain. The present set of experiments merely shows that brain has the enzymes required for urea synthesis, but does not indicate what role they play in brain metabolism. Thus, for the present, a definitive and comprehensive picture of the manner in which the brain handles the highly toxic substance ammonia is still lacking, though one may anticipate that in the near future there will be an integration of the roles of the enzymes of the glutamic acid–glutamine system and of the enzymes participating in urea synthesis.

SUMMARY

Evidence that the living rat brain can convert L-arginine to urea is presented. The implications of this finding with respect to (1) urea synthesis in brain and (2) ammonia detoxication in brain are discussed.

Acknowledgements—We wish to express our deepest appreciation to Dr. JOHN ROMANO and Dr. GEORGE L. ENGEL for their devoted and generous support and encouragement of these investigations. We are indebted to Dr. DONALD B. TOWER for criticism of the manuscript. Dr. TOWER, Dr. J. J. PISANO, and Dr. SIDNEY UDENFRIEND kindly supplied us with a sample of γ-guanidinobutyric acid. Miss AILENE WILLIAMS contributed valuable technical assistance.

REFERENCES

BACH S. J. and SMITH M. (1956) *Biochem. J.* **64,** 417.

BALDWIN E. (1957) *Dynamic Aspects of Biochemistry* (3rd Ed.) p. 308. Cambridge University Press.

VOL.
5
1959/6

Bollman J. L., Mann F. C., and Magath T. B. (1924) *Amer. J. Physiol.* **69,** 371.
Bronk J. R. and Fisher R. B. (1956) *Biochem. J.* **64,** 106, 111, 118.
Cohen P. P. (1954) In *Chemical Pathways of Metabolism* (Edited by Greenberg D. M.) Vol. 2, p. 1. Academic Press, New York.
Connerty H. V., Briggs A. R. and Eaton E. H. (1955) *Amer. J. Clin. Path.* **25,** 1321.
Engel M. G. and Engel F. L. (1947) *J. biol. Chem.* **167,** 535.
Geiger A., Magnes J., Taylor R. M. and Veralli M. (1954) *Amer. J. Physiol.* **177,** 138.
Geiger A. and Yamasaki S. (1956) *J. Neurochem.* **1,** 93.
Krebs H. A. and Henseleit K. (1932) *Hoppe-Seyl. Z.* **210,** 33.
Miller L. L., Burke W. T. and Haft D. E. (1956) In *Some Aspects of Amino Acid Supplementation* (Edited by Cole W. H.) p. 44. Rutgers Univ. Press, New Brunswick, N. J.
Ratner S. (1954) *Advanc. Enzymol.* **15,** 319.
Rosenberg H., Ennor A. H. and Morrison J. F. (1956) *Biochem. J.* **63,** 153.
Sharpe L. M., Culbreth G. G. and Klein J. R. (1950) *Proc. Soc. Exp. Biol., N. Y.* **74,** 681.
Sporn M. B., Dingman W. and Defalco A. (1959) *J. Neurochem.* **4,** 141.
Strecker H. J. (1957) In *Metabolism of the Nervous System* (Edited by Richter D.) p. 259. Pergamon Press, New York.
Tallan H. H., Moore S. and Stein W. H. (1954) *J. biol Chem.* **211,** 927.

OL.
5
9/60

Journal of Neurochemistry, 1959, Vol. 5, pp. 68 to 72. Pergamon Press Ltd. Printed in Northern Ireland

DISTRIBUTION OF CATECHOL-O-METHYL TRANSFERASE IN THE NERVOUS SYSTEM AND OTHER TISSUES

JULIUS AXELROD, WAYNE ALBERS and CARMINE D. CLEMENTE*
Laboratory of Clinical Science, National Institute of Mental Health
and
Laboratory of Neuroanatomical Science, National Institute of Neurological Diseases and Blindness, Bethesda, Maryland

(*Received* 8 *April* 1959)

VOL. 5 1959/6

O-METHYLATION has been shown to be the principal pathway for the metabolism of administered adrenaline and noradrenaline (AXELROD, INSCOE, SENOH and WITKOP, 1958; LABROSSE, AXELROD and KETY, 1958). An enzyme, catechol-O-methyl transferase (COMT),† which catalyses the transfer of a methyl group from S-adenosylmethionine to the 3-hydroxy group of catechols, has been described (AXELROD 1957; AXELROD and TOMCHICK, 1958). Since the products, metanephrine (3-O-methyladrenaline) and normetanephrine (3-O-methylnoradrenaline), have negligible physiological activity (EVARTS *et al.*, 1958), this enzyme may be of major importance in the inactivation of endogenously released catecholamine hormones. The functional role of catecholamines in the autonomic nervous system and the relatively high concentration of these amines in certain regions of the central nervous system (VOGT, 1954) suggested a study of the distribution of COMT in the nervous system and certain organs.

METHODS AND MATERIALS

Preparation of tissues. Tissues were obtained from adult monkeys (*Macaca mulatta*), which were killed by exsanguination under pentobarbital anaesthesia. The tissues were dissected immediately and cooled in crushed ice. When samples were prepared for enzyme assay, all operations were carried out at 0–4°. Tissues were homogenized with 5 vol of isotonic KCl solution and centrifuged at 14,000 g for 20 min.

Enzyme assay. An aliquot of the soluble supernatant fraction was taken for enzyme assay (AXELROD and TOMCHICK, 1958). COMT activity was determined by measuring the amount of ^{3}H-metanephrine formed from ^{3}H-adrenaline. The incubation mixture, in a 10 ml glass-stoppered centrifuge tube, contained 7 μmoles phosphate buffer, pH 7·8, 0·5 μmole $MgCl_2$, 20 mμmoles AMe, 2·8 mμmoles *dl*-β-^{3}H-adrenaline bitartrate (260 mμc), and 25 μl enzyme preparation in a final volume of 100 μl. Blank values were obtained by omitting AMe from the above mixture. After incubation for 90 min at 37°, 0·5 ml of 0·5 M-borate buffer, pH 10, and 5 ml of a mixture of toluene–isoamyl alcohol (3 : 2) were added to the reaction mixture, and the tube was shaken mechanically for 15 min. After centrifuging, a 3 ml aliquot of the organic solvent phase was added to a mixture of 1 ml ethanol and 5 ml of 0·4% 3:4-diphenyloxazole and 0·01% β-bis-[2-(phenyloxazolyl)] benzene in toluene and counted in a liquid scintillation spectrometer. All values were corrected for the partition of metanephrine in the above solvent-buffer system.

* Visiting Scientist, present address: Department of Anatomy, University of California Medical School, Los Angeles, California.

† Abbreviations used in this paper are as follows:
COMT = Catechol-O-methyl transferase.
ATP = Adenosine triphosphate.
AMe = S-Adenosylmethionine.
Tris = Tris(hydroxymethyl)aminomethane.

Specificity of the method. The material extracted from the incubation mixture at pH 10·0 into the toluene–isoamyl alcohol mixture showed the presence of single radioactive peaks on Whatman No. 1 paper after chromatography in butanol : acetic acid : water (8 : 2 : 2) or isopropanol : 5% ammonia (8 : 2). These peaks had the same R_F values as authentic metanephrine.

RESULTS

Distribution of COMT

(Table 1). Highest enzyme activity was found in the liver of the monkey: it was considerably greater than in any of the species previously examined (AXELROD and TOMCHICK, 1958). In monkey brain, COMT activity was several times higher than that found for rat brain.

Several glandular tissues were found to have relatively high enzyme activity. The magnitude of COMT activities in thyroid gland, spleen, blood vessels, and peripheral nerves were similar to that found in the brain. COMT activity was present in all peripheral nerves and in all areas of the brain which were examined. Within the central nervous system, a four-fold range of enzyme activity was found, lowest values occurring in the cerebellar cortex and amygdala, whereas the highest were found in the neurohypophysis.

The noradrenaline levels reported for different regions of dog brain (VOGT, 1954) bear no direct relationship to the COMT activities found in monkey brain. Although negligible amounts of noradrenaline have been reported to be present in fibre tracts, the activity of COMT was relatively high in optic tract and corpus callosum. No simple correlation between total cell density and enzyme activity is apparent, since gray areas of such differing cell populations as occipital cortex and hypothalamus had similar enzyme activities. Furthermore, there was no localization of the enzyme in highly vascular regions of the brain. The possibility remains that COMT activity may be associated with a particular cell type which is not evident at the level of histochemical localization attained in this study.

Enzyme activity in the sympathetic ganglion (superior cervical) was found to be twice as great as that in the post-ganglionic sympathetic fibres. Comparable enzyme activity was present in parasympathetic, motor, and sensory nerves.

Synthesis of S-adenosylmethionine in the nervous system. O-Methylation of catecholamines *in vivo* is dependent not only upon the occurrence of COMT in the tissue, but also upon the ability of the tissue to form the methyl donor, AMe (CANTONI, 1953). In view of the potential importance of O-methylation in the nervous system, the ability of the brain to synthesize AMe was investigated. The results shown in Table 2 definitely establish the enzymic synthesis of AMe from ATP and methionine by brain tissue. The lower activity of COMT in this experiment was due to the presence of tris buffer, which is optimal for the methionine activating enzyme, but somewhat inhibitory for COMT.

DISCUSSION

The physiological actions of catecholamines at many different kinds of peripheral receptors, their participation in metabolic reactions, and their release from sympathetic nerve endings are well established; but the role of these amines in the central nervous system is poorly understood. Until recently, monoamine oxidase was considered to be the enzyme principally involved in the inactivation of catecholamines. However, it has been shown that inhibition of this enzyme with iproniazid does not affect the

TABLE 1.—CATECHOL-O-METHYL TRANSFERASE ACTIVITY IN TISSUES OF MONKEY

	Metanephrine formed per gram of tissue in 90 min* (μmoles)
Central nervous system:	
Neurohypophysis	1·46
Sup. and inf. colliculi	0·96
Area postrema	0·94
Optic tract	0·92
Ventral midbrain	0·83
Glob. pallidus and putamen	0·82
Corpus callosum	0·82
Pons	0·79
Caudate nucleus	0·78
Ventral medulla	0·75
Occipital cortex	0·73
Hypothalamus	0·73
Dorsal hippocampus	0·70
Spinal dorsal funiculus	0·65
Precentral gyrus, cerebral cortex	0·58
Ventral hippocampus	0·46
Amygdala	0·37
Cerebellar cortex (ant. lobe)	0·34
Peripheral nervous system:	
Superior cervical ganglion	0·99
Vagus nerve	0·97
Saphenous nerve	0·69
Splanchnic nerve	0·67
Sciatic nerve	0·62
Sacral sympathetic nerve fibers	0·50
Splenic nerves	0·46
Other tissues:	
Liver	13·0
Submaxillary gland	6·3
Parotid	5·4†
Pancreas	5·2
Adenohypophysis	3·2
Thyroid	0·9
Spleen	0·6
Aorta	0·9
Inf. vena cava	0·6

* Results are the mean values from tissues obtained from three to five monkeys.

† Value for one animal.

VOL. 5 1959/6

TABLE 2.—OCCURRENCE OF METHIONINE ACTIVATING ENZYME IN MONKEY BRAIN

Soluble supernatant fraction obtained from 160 mg of monkey brain was incubated with 0·2 ml $MgCl_2$ solution, 0·5 M; 0·2 ml M-tris buffer, pH 7·4; 6 μmoles adrenaline bitartrate, and added cofactors in a final volume of 1·5 ml. After incubation at 37° for 90 min, the mixture was assayed for metanephrine by a procedure previously described (AXELROD and TOMCHICK, 1958).

Cofactors added	Metanephrine formed per g brain tissue (μmoles)
None	0·00
ATP, 10 μmoles	0·08
Methionine, 10 μmoles	0·00
ATP, Methionine, 10 μmoles each	0·22
AMe, 0·4 μmole	0·20

duration or intensity of action of exogenous or endogenous catecholamines (GRIESEMER *et al.*, 1953; BROWN and GILLESPIE, 1957). Furthermore, monoamine oxidase acts primarily in the deamination of the metabolites of adrenaline and noradrenaline rather than on the catecholamines directly (AXELROD, INSCOE, SENOH and WITKOP, 1958); LABROSSE, ALEXROD and KETY, 1958). Recent work has demonstrated that part of administered adrenaline in the whole animal persisted long after the physiological effects were gone (AXELROD and TOMCHICK, unpublished observation). This suggests that initial inactivation might occur by two processes: binding of the intact molecule (e.g. to protein), and enzymic O-methylation.

O-methylation has been shown to be the principal pathway for the metabolism of administered catecholamines (AXELROD, INSCOE, SENOH and WITKOP, 1958; LABROSSE, AXELROD and KETY 1958). This finding strongly implicates COMT as the enzyme chiefly concerned with the metabolism of circulating catecholamines. Normetanephrine and metanephrine also occur normally in tissues rich in catecholamines as well as in the urine (AXELROD, 1957; AXELROD, SENOH and WITKOP, 1958). Preliminary results from this laboratory indicate that the K_m of brain COMT for adrenaline is less that 10^{-5} M, which places it in the range of endogenous catecholamine concentration. All of these observations support the possiblity that COMT is a major enzyme in the inactivation of endogenous catecholamines.

SUMMARY

Catechol-O-methyl transferase (COMT), an enzyme involved in the metabolism of adrenaline, noradrenaline, and other catechols, has been shown to be present throughout the central nervous system, as well as in sensory, motor, parasympathetic, and sympathetic nerves, glandular tissues, and blood vessels. Methionine-activation, required for the synthesis of the cofactor for COMT, was also shown to occur in brain tissue. The possible role of COMT in the metabolism of catecholamines is discussed.

REFERENCES

AXELROD J. (1957) *Science* **126**, 400.
AXELROD J., INSCOE J. K., SENOH S. and WITKOP B. (1958) *Biochim. Biophys. Acta* **27**, 210.
AXELROD J., SENOH S. and B. WITKOP (1958) *J. biol. Chem.* **233**, 697.

AXELROD J. and TOMCHICK R. (1958) *J. biol. Chem.* **233,** 702.
AXELROD J. and TOMCHICK R. (unpublished observations).
BROWN G. L. and GILLESPIE S. S. (1957) *J. Physiol.* **138,** 81.
CANTONI G. L. (1953) *J. biol. Chem.* **204,** 403.
EVARTS E. V., GILLESPIE L., FLEMING T. E. and SJOERDSMA A. (1958) *Proc. Soc. exp. Biol., N.Y.* **98,** 74.
GRIESEMER E. C., BARSKY C. A., DRAGSTEDT C. A., WELLS J. A. and ZELLER E. A. (1953) *Proc. Soc. exp. Biol., N.Y.* **84,** 699.
LABROSSE E. A., AXELROD J. and KETY S. S. (1958) *Science* **128,** 593.
VOGT M. (1954) *J. Physiol.* **123,** 451.

VOL.
5
1959/6

Journal of Neurochemistry, 1959, Vol. 5, pp. 73 to 79. Pergamon Press Ltd. Printed in Northern Ireland

OXIDATION OF *p*-PHENYLENEDIAMINE AND ADRENALINE IN ENZYMIC AND COPPER-CATALYSED REACTIONS

EDWARD GELLER, SAMUEL EIDUSON and ARTHUR YUWILER*
Neurobiochemistry Laboratory, Veterans Administration Center and Departments of Physiological Chemistry, Psychiatry, and Anatomy, Medical Center, University of California, Los Angeles

(*Received* 21 *February* 1959)

OL.
5
9/60

THE study of the oxidation of amines by serum has taken two directions. One, involving the measurement of the oxygen consumed in reactions of this type, is characterized by the studies of the enzyme, ceruloplasmin (HOLMBERG and LAURELL, 1948, 1951*a*, *b*). The second, based upon colour formation, is mainly concerned with whole serum and the amines, *p*-phenylenediamine, N,N-dimethyl-*p*-phenylenediamine, and adrenaline (LEACH and HEATH, 1956; LEACH *et al.*, 1956; AKERFELDT, 1957*a*, *b*; ABOOD, 1957). Of late, these two lines have merged, with the suggestion that the colour forming component of serum could be ceruloplasmin (LEACH *et al.*, 1956).

Recent reports (subsequently disputed) that the blood of schizophrenics is high in oxidase activity (AKERFELDT, 1957*a*, *b*; ABOOD, 1957; BROWN, 1957; ANGEL *et al.*, 1957; ABOOD *et al.*, 1957) stimulated in this laboratory a study of the properties of ceruloplasmin. In the course of this investigation, findings contrary to some of the published results came to light and they are reported below.

MATERIALS AND METHODS

Serum. Human serum for testing was obtained by drawing 20 ml of blood from the antecubital vein, allowing it to clot, and centrifuging. Human serum for the isolation of ceruloplasmin was obtained by dialysing outdated blood bank serum to remove the citrate and dextrose. Retroplacental serum was obtained at the UCLA Medical Center from a patient in delivery. The blood, contaminated with amniotic fluids, was centrifuged.

Pig serum was obtained at the slaughterhouse: the blood was collected in a large stainless steel pail and allowed to clot during transportation to the laboratory (about 1 hr).

Ceruloplasmin. The procedure of HOLMBERG and LAURELL (1948) was followed with the exception that occasionally the chloroform–alcohol extraction was replaced by repeated ammonium sulphate fractionations at 42·5 to 47·5% saturation. Details of a typical preparation are given in Table 1. It can be calculated from the data of HOLMBERG and LAURELL (1948) that purified ceruloplasmin contains 21 μg Cu/mg N, so that our preparation still contained extraneous protein. Paper electrophoresis confirmed this calculation, for the final preparation, although apparently homogeneous at pH 8·6, showed two distinct bands at pH 7·4.

In order to follow the purification, an arbitrary unit was defined. The colorimetric estimation of oxidase activity reported by ABOOD *et al.* (1957), which employs *p*-phenylenediamine as substrate, was used. Test specimens were incubated for 0·5 hr rather than 1 hr as recommended by Abood as it was found that the ascorbic acid level had no effect with 0·5 hr incubation. One unit of activity was defined as that quantity which gave a change of optical density of 0·01 at 490 mμ when incubated

* Present address: Schizophrenia & Psychopharmacology Joint Research Project, Box A, Ypsilanti, Michigan.

at 37° for 0·5 hr with 0·5 ml of 0·1% *p*-phenylenediamine solution buffered at pH 6·8 with tris-hydroxymethylaminomethane (tris).

Substrates. *p*-Phenylenediamine was obtained from Distillation Products Industries; L-adrenaline bitartrate and L-noradrenaline were products of Winthrop-Stearns; DL-3:4-dihydroxyphenylalanine, serotonin creatinine sulphate, β-(3:4-dihydroxyphenyl)ethylamine (dopamine), tyramine hydrochloride, γ-aminobutyric acid, (GABA) and histamine diphosphate were Cfp grade products of the California Corporation for Biochemical Research. $CuSO_4 \cdot 5H_2O$ was Mallinckrodt AR grade.

Methods. Conventional Warburg methods were employed to measure oxygen uptake. Reactions were started by tilting substrates from the side arms of 20 ml Warburg flasks into the main compartment containing enzyme and buffer. In some runs copper sulphate solution was tilted from a second side arm at the same time. Both acid and alkali center wells were employed. In some experiments the system was flushed with oxygen before tilting.

Copper was determined by the method of GUBLER *et al.* (1952).

RESULTS

p-Phenylenediamine was readily oxidized in 0·1 M-acetate buffer, pH 6·0, by all the preparations used (Fig. 1). The most active ceruloplasmin fraction obtained (alcohol-chloroform extract, Table 1), although still quite impure, exhibited a rapid oxygen

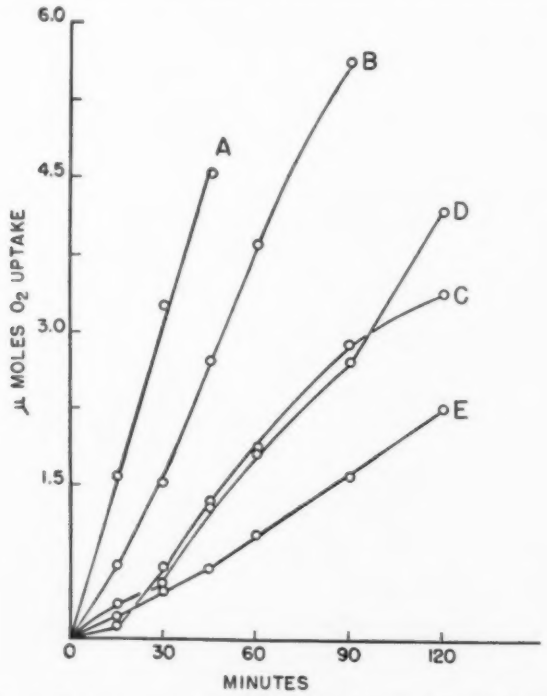


FIG. 1.—The ordinates represent μmoles oxygen taken up by a solution containing 0·5 ml of a *p*-phenylenediamine solution (10 μmoles), the solutions indicated below, and sufficient 0·1 M-tris buffer, pH 6·0, to make a final volume of 3·5 ml.

Curve A. 1·0 ml of alcohol–chloroform extract of pig serum (fraction 4a, Table 1).
Curve B. 1·0 ml retroplacental serum, after 24 hr dialysis against running tap water.
Curve C. 1·0 ml of alcohol fraction of blood bank serum (equivalent to fraction 3, Table 1).
Curve D. 1·0 ml of retroplacental serum.
Curve E. 1·0 ml of blood bank serum.

VOL. 5 1959/6

TABLE 1.—FRACTIONATION OF PIG SERUM PROTEINS

Fraction	Total units*	Units/mg N	μg Cu/mg N	Recovery of units (%)
1. Whole serum	$4{\cdot}42 \times 10^5$	14·2	0·11	—
2. 36–55% $(NH_4)_2SO_4$	$1{\cdot}55 \times 10^5$	46·1	0·35	35·2
3. 16% alcohol	$4{\cdot}96 \times 10^4$	65·4	0·79	11·3
4a. Alcohol–chloroform extract	$4{\cdot}21 \times 10^3$	532·8	3·52	0·9
4b. 42·5–47·5% $(NH_4)_2SO_4$†	$7{\cdot}86 \times 10^3$	364·3	3·06	1·7

* One unit is defined as that quantity of oxidase activity which gives a change of optical density of 0·01 at 490 mμ when incubated at 37° for 0·5 hr with 0·5 ml of 0·1% *p*-phenylenediamine solution buffered at pH 6·8 with tris.

† This fractionation procedure was repeated three times.

uptake with *p*-phenylenediamine (Curve A, Fig. 1). The preparation was clearly similar to that of HOLMBERG and LAURELL (1948); it had a deep blue colour and migrated as an α-2-globulin in an electrophoretic field. At pH 6·0, the pH at which maximal activity was reached, the K_m of the reaction of this fraction with *p*-phenylenediamine was $1{\cdot}9 \times 10^{-3}$ moles/litre, in good agreement with the reported value of $2{\cdot}5 \times 10^{-3}$ (HOLMBERG and LAURELL, 1951*a*). In spite of the apparent similarity of the two preparations, DOPA and adrenaline did not appear to be substrates for the enzyme, as had been reported (HOLMBERG and LAURELL, 1951*a*). Similarly, no oxygen uptake was found with serotonin, dopamine, tyramine, GABA, or histamine as substrates and the various serum preparations listed above as the source of enzyme activity, as has been reported (MARTEN *et al.*, 1958; PORTER *et al.*, 1957). A possible explanation of these findings might lie in the fact that in most of the studies in which oxidation with these amines was reported either dilute buffers (MARTEN *et al.*, 1958; PORTER *et al.*, 1957) were used or no buffer at all (HOLMBERG and LAURELL, 1951*a*).

The oxidation of adrenaline was then investigated. At pH 6·0 a $2{\cdot}9 \times 10^{-3}$ M-adrenaline solution in 0·04 M-tris-HCl buffer remained unchanged for long periods of time. After three hours at 35° no oxygen uptake was observed and only the faintest trace of yellowing was noted. Similar results were obtained in 0·1 M-tris-HCl buffer. If $CuSO_4$ was added to the buffer solutions to a concentration of 10^{-5} M, a rapid oxygen uptake was observed with the dilute buffer, but not with the more concentrated one (Fig. 2). Colour formation paralleled the oxygen uptake. At the conclusion of the run the pH had become alkaline in the experimental flasks (pH range 7·1–7·7) containing the 0·04 M-buffer, but remained at pH 6·0 in the controls to which no copper had been added and in the flasks containing the 0·1 M-buffer. When serum or serum preparations were added to these solutions (at pH 6·0) no oxygen uptake was found but a considerable amount of red colour appeared.

At pH 7·4, a $2{\cdot}9 \times 10^{-3}$ M-adrenaline solution in either 0·04 M- or 0·1 M-tris-HCl buffer took up some oxygen and developed a pink colour (Fig. 3). When the solutions also contained 10^{-5} M-$CuSO_4$, oxygen uptake was rapid and deep red colours developed. If, instead of $CuSO_4$, serum or enzyme preparation was added to the adrenaline-buffer solutions, a small increase in oxygen uptake above that measured

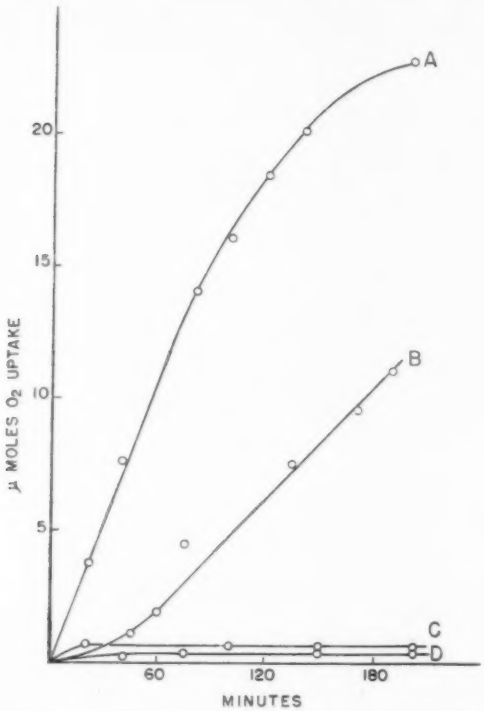


FIG. 2.—The ordinates represent μmoles oxygen taken up by a flask containing 0·5 ml of adrenaline solution (10 μmoles), $3{\cdot}5 \times 10^{-2}$ μmoles of $CuSO_4$, and the solutions indicated below:

Curve A. Sufficient 0·04 M-tris buffer, pH 6·0, to make a final volume of 3·5 ml.
Curve B. As A, but with 0·5 ml of pig serum added.
Curve C. As A, but with 0·5 ml of bovine albumin solution (3·50 g/100 ml) added.
Curve D. Sufficient 0·1 M-tris buffer, pH 6·0 to make a final volume of 3·5 ml.

with the buffer alone was seen, and a considerable amount of colour was formed. When, however, serum was added to the solutions which contained copper, colour formation and oxygen uptake were markedly diminished. Albumin also inhibited this reaction, but to a lesser extent (Fig. 3).

DISCUSSION

A summary of the results is presented in Table 2. At pH 7·4 no additions were needed to induce colour formation or oxygen uptake. This was most probably caused by trace metal impurities in the solutions. It has been observed previously (CHAIX *et al.*, 1950; VARÈNE, 1957*a*) that this trace metal catalysis is pH dependent and no effect is found at pH 6·0. The addition of copper to the solution at pH 6·0 induced the formation of some colour. This was probably caused by the formation of the copper-adrenaline complex demonstrated by CHAIX *et al.* (1950). The rate of the further reaction of this complex with oxygen is many times greater at pH 7·4 than at pH 6·0 (CHAIX *et al.*, 1950; VARÈNE, 1957*a*) so that vigorous oxygen uptake was

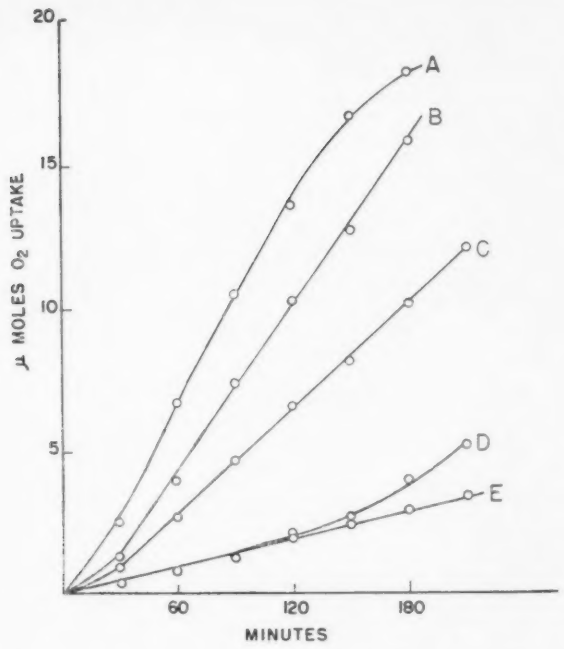


FIG. 3.—The ordinates represent μmoles of oxygen taken up by a flask containing 0·5 ml of adrenaline solution (10 μmoles), $3{\cdot}5 \times 10^{-2}$ μmoles of $CuSO_4$ (except E), and the solutions indicated below:

Curve A. Sufficient 0·1 M-tris buffer, pH 7·4, to make a final volume of 3·5 ml.
Curve B. As A, but with 0·5 ml of bovine albumin solution (3·50 g/100 ml) added.
Curve C. As A, but with 0·5 ml of alcohol fraction of pig serum (Fraction 3, Table 1) added.
Curve D. As A, but with 0·5 ml of pig serum added.
Curve E. As A, but containing no $CuSO_4$.

seen at the alkaline pH. Along with the oxygen uptake, the appearance of the red colour characteristic of adrenochrome (BOUVET, 1949) was observed. However, at pH 6·0 no further reaction of the complex was seen. When serum (or enzyme preparations, or albumin) was added to the system at pH 7·4 an inhibition of the copper catalysed oxidation was seen. This has been observed before (VARÈNE, 1957*b*; WALAAS and JERVELL, 1958) and it is generally considered to result from the binding of copper by protein. When, however, serum was added to the system at pH 6·0 an acceleration of the colour production, but not of oxygen uptake, resulted. This was probably due to a coupled reaction between the adrenaline-copper complex and some reducible component of serum. The suggestion that this coupling is enzymic is unnecessary: a copper-catalysed electron transfer could proceed non-enzymically. The fact that heat-treated serum has lost this ability to activate (PAYZA and HOFFER, personal communication) does not necessarily imply an enzyme, because heat coagulation will effect other components in serum as well as proteins.

The colour which appeared at pH 6·0 when serum and adrenaline were mixed without copper can be explained by the copper present in the serum and the trace

TABLE 2.—SUMMARY OF RESULTS ON THE OXIDATION OF ADRENALINE

Solution	pH 6·0		pH 7·4	
	Colour	O_2 uptake	Colour	O_2 uptake
Buffer A or B	None	None	Pink	*
Buffer B + $CuSO_4$	Yellow-pink	None	Dark Red	‡
Buffer A + $CuSO_4$	Red	‡	Dark Red	‡
Buffer B + Serum	Faint pink	None	Red	†
Buffer B + Serum + $CuSO_4$	Red	None	Dark Red	*

Flasks contained 0·5 ml. of adrenaline solution at the appropriate pH (10 μmoles/0·5 ml), 0·5 ml serum where indicated, 0·2 ml $CuSO_4$ solution at the appropriate pH (1·75 μmoles/ml) where indicated, and sufficient buffer A or B to make a total of 3·5 ml. Buffer A was 0·04 M-tris-HCl, pH 6·0 or pH 7·4 (as indicated). Buffer B was 0·1 M-tris-HCl, pH 6·0 pr pH 7·4 (as indicated).

* 0 to 3 μmoles oxygen uptake in 2 hr incubation at 35°.
† 3 to 7·5 μmoles oxygen uptake in 2 hr incubation at 35°.
‡ over 7·5 μmoles oxygen uptake in 2 hr incubation at 35°.

VOL. 5 1959/6

amounts possibly present in the water used. This amount of copper (the concentration of copper in serum is about $1{\cdot}6 \times 10^{-5}$ M, much of it associated with proteins) is sufficient to cause a rapid reaction at pH 7·4, and serum alone will produce colour in adrenaline solutions at pH 7·4. However, in this case oxygen uptake is considerably less than expected, possibly due to the presence of an electron acceptor competing with atmospheric oxygen.

Colour formation in these systems is, as has frequently been indicated, the result of a complex series of reactions. Initiated by metal activation of the amine (involving an enzyme or not), the reaction proceeds to highly polymerized, structurally unidentified compounds at unknown oxidation levels. There appears to be no true correlation between the intensity of the colour produced in these reactions and the extent of the oxidation.

SUMMARY

1. Oxidation of *p*-phenylenediamine was catalysed by serum and ceruloplasmin preparations. The optimal pH was 6·0 and the K_m of the reaction was $1{\cdot}9 \times 10^{-3}$.

2. At pH 6·0 neither serum nor ceruloplasmin preparations showed measurable oxygen uptake with adrenaline, noradrenaline, serotonin, dopamine, tyrosine, GABA, or histamine. Colour formation was, however, frequently noted.

3. When 10^{-5} M-cupric ion was added to the buffers, rapid acceleration of oxygen uptake was found with adrenaline at pH 7·4. When cupric ions were added to buffer of pH 6·0 no uptake was observed unless dilute buffers were used. In these experiments, the pH changed during the course of the run. When the pH became alkaline, oxygen uptake was observed.

4. Ceruloplasmin, serum, and bovine albumin inhibited the copper-catalysed oxidation of adrenaline at pH 7·4. Serum induced the appearance of colours at pH 6·0.

5. It is suggested that serum does not oxidize adrenaline enzymically and that

the observed oxygen uptake derives from metal-catalysed autoxidation at alkaline pHs.

6. Colour formation is not a reliable indicator of enzymic oxidation in these systems.

REFERENCES

ABOOD L. G. (1957) *Blood Tests in Mental Illness.* Papers and Discussion presented at the Annual Scientific Conference of the Brain Research Foundation, Chicago, p. 12.

ABOOD L. G., GIBBS F. A. and GIBBS E. (1957) *Arch. Neurol. Psychiat., Chicago* **77,** 643.

AKERFELDT S. (1957*a*) *Blood Tests in Mental Illness.* Papers and Discussion presented at the Annual Scientific Conference of the Brain Research Foundation, Chicago, p. 6.

AKERFELDT S. (1957*b*) *Science* **125,** 117.

ANGEL C., LEACH B. E., MARTENS S., COHEN M. and HEATH R. G. (1957) *Arch. Neurol. Psychiat., Chicago* **78,** 500.

BOUVET P. (1949) *Bull. Soc. Chim. Biol., Paris* **31,** 1301.

BROWN B. S. (1957) *J. Pediat.* **51,** 46.

CHAIX P., CHAUVET J. and JEZEQUEL J. (1950) *Biochim. biophys. Acta* **4,** 471.

GUBLER C. J., LAHEY M. E., ASHENBRUCKER H., CARTWRIGHT G. E. and WINTROBE M. M. (1952) *J. biol. Chem.* **196,** 209.

HOLMBERG C. G. and LAURELL C. B. (1948) *Acta chem. scand.* **2,** 550.

HOLMBERG C. G. and LAURELL C. B. (1951*a*) *Acta chem. scand.* **5,** 476.

HOLMBERG C. G. and LAURELL C. B. (1951*b*) *Scand. J. clin. Lab. Invest.* **3,** 103.

LEACH B. E. and HEATH R. G. (1956) *Arch. Neurol. Psychiat., Chicago* **76,** 444.

LEACH B. E., COHEN M., HEATH R. G. and MARTENS S. (1956) *Arch. Neurol. Psychiat., Chicago* **76,** 635.

MARTEN G., ERIKSEN N. and BENDITT E. P. (1958) *Fed. Proc.* **17,** 447.

PORTER C. C., TITUS D. C., SAUNDERS B. E. and SMITH E. V. C. (1957) *Science* **126,** 1014.

VARÈNE P. (1957*a*) *Bull. Soc. Chim. biol., Paris* **39,** 1099.

VARÈNE P. (1957*b*) *Bull. Soc. Chim. biol., Paris* **39,** 1473.

WALAAS E. and JERVELL K. F. (1958) Abstract from Fourth International Congress of Biochemistry. *Int. Abstr. biol. Sci.* Suppl. p. 106.

Journal of Neurochemistry, 1959, Vol. 5, pp. 80 to 90. Pergamon Press Ltd. Printed in Northern Ireland

GLUTAMIC ACID AND GLUTAMINE METABOLISM IN CEREBRAL CORTEX AFTER SEIZURES INDUCED BY METHIONINE SULPHOXIMINE

EDMUND L. PETERS and DONALD B. TOWER
Section of Clinical Neurochemistry,
National Institute of Neurological Diseases and Blindness,
Bethesda, Maryland, U.S.A.

(*Received* 4 *March* 1959)

VOL.
5
1959/6

METHIONINE sulphoximine is the toxic principle isolated from flour treated by the nitrogen trichloride, or 'Agene', process (BENTLEY *et al.*, 1950; MELLANBY, 1946, 1947; MISANI and REINER, 1950). It is a potent convulsant for all mammalian species tested, producing a semi-chronic seizure state within 12 to 24 hours after oral or parenteral administration (REINER *et al.*, 1950; TOWER, 1958*a*). It has also been shown to inhibit the growth of various microorganisms and to be counteracted in this respect by addition of methionine or glutamine to the culture media (HEATHCOTE and PACE, 1950; NEWELL and CARMAN, 1950). Inhibition of cerebral glutamine synthesis by methionine sulphoximine (PACE and MCDERMOTT, 1952) appears to be closely analogous to similar inhibition by methionine sulphoxide in a variety of tissue preparations (BOREK *et al.*, 1946; ELLIOTT and GALE, 1948; SPECK, 1949). The seizures induced by methionine sulphoximine can be prevented or arrested by *in vivo* administration of large doses of methionine, glutamine, or asparagine (REINER *et al.*, 1950; TOWER, 1959*b*). Cerebral cortex slices from cats with such seizures exhibit during incubation *in vitro* a failure of 'bound' acetylcholine production, which can be corrected by *in vitro* addition of methionine, glutamine, or asparagine (TOWER, 1959*b*; TOWER and ELLIOTT, 1953). These various observations suggest that methionine sulphoximine acts both as a methionine antagonist or antimetabolite and as an inhibitor of glutamine metabolism. It is not apparent whether or not the two effects are directly interrelated, nor is the exact mode of action of methionine sulphoximine clear. The studies reported here on metabolism of glutamic acid and glutamine in incubated slices of cat cerebral cortex were undertaken to obtain further information on these points, since disturbances of glutamic acid metabolism appear to be common to many different seizure states (TOWER, 1959*b*). Some preliminary accounts have been previously reported (TOWER, 1957*b*; 1959*b*).

MATERIALS AND METHODS

All studies were carried out on stock, adult cats. DL-methionine sulphoximine was generously supplied by the late Dr. L. REINER of Wallace and Tiernan Co., Inc., Belleville, New Jersey. Reagent grade L-amino acids were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and adenosine triphosphate sodium (ATP) from Sigma Chemical Co., St. Louis, Missouri. Analytically pure 2-pyrrolidinone and pyridoxal phosphate were kindly donated by Dr. L. SARETT of Merck-Sharpe and Dohme Research Laboratories, Rahway, New Jersey. All other chemicals were of reagent grade, obtained from usual commercial sources.

Seizures were induced in cats by intraperitoneal injection of 0·1 m-moles (15–20 mg/kg body weight) of methionine sulphoximine dissolved in 2 ml of 0·9% NaCl solution. With this dose seizures

regularly occurred within 18–24 hr. No experimental animal was sacrificed until at least one generalized seizure had been personally observed. The unanaesthetized seizure and normal cats were sacrificed by decapitation, and cerebral cortex slices were prepared and incubated as previously described (TOWER, 1958*b*). All incubations were carried out with a bicarbonate–saline medium containing glucose, 10 mM, and K^+, 27 mM, under conditions previously outlined in detail (TOWER, 1958*b*). All *in vitro* additions were to a final concentration of 10 mM, except sodium ATP which was to 3 mM and pyridoxal phosphate which was to 1 mM.

Glutamic acid, glutamine, and ammonia contents of slices (and in some cases of incubation media) were determined by the *Clostridium perfringens* method for total glutamate (MEISTER *et al.*, 1951) combined with microdiffusion and spectrophotometric analyses for free and amide ammonia (RUSSELL, 1944), previously reported in detail (TOWER, 1958*b*). Determinations of pre-formed or true γ-aminobutyric acid content of slices and of total γ-aminobutyric acid after bacterial decarboxylation of glutamate were carried out through the co-operation of Dr. G. M. MCKHANN using his unpublished modification of the fluorimetric procedure of LOWE *et al.* (1958). All values, including incubation media content, have been expressed as μmoles/g fresh tissue.

RESULTS AND DISCUSSION

Normal vs. epileptogenic samples. Glutamate metabolism by slices from methionine sulphoximine-intoxicated cats compared to that by slices from normal cats is summarized in Table 1. Normal cortical slices exhibited significant increases in levels

TABLE 1.—GLUTAMATE METABOLISM IN INCUBATED SLICES OF CEREBRAL CORTEX FROM NORMAL CATS AND FROM CATS WITH SEIZURES INDUCED BY METHIONINE SULPHOXIMINE
(μmoles/g)

Component	Incub. time (min)	Normal		Methionine sulphoximine	
		Level	Change	Level	Change
Glutamic Acid (17/13)#	0	7·5 (±0·8)		6·45(±1·0)‡	
	60	9·55(±0·9)	+2·15(±0·85)*	5·5(±1·05)*	−0·95(±0·75)†
Glutamine (17/13)	0	3·2 (±0·85)		0·1 (±0·05)*	
	60	5·35(±1·0)	+2·2 (±1·0)*	0·55(±0·35)*	+0·35(±0·45)*
γ-Aminobutyric Acid (13/2)	0	8·6 (±1·05)		7·35(±1·75)	
	60	9·7 (±0·95)	+1·1 (±0·5)†	5·3 (±1·6)‡	−2·0 (±0·2)
Tissue Ammonia (14/10)	0	1·95(±0·5)		2·0 (±0·55)	
	60	2·2 (±0·55)	+0·3 (±0·35)	2·5 (±0·4)	+0·55(±0·45)
MEDIUM Glutamate (14/3)	0	5·3 (±1·05)		1·3 (±0·15)*	
	60	2·9 (±0·85)	−2·25(±0·9)*	0·0 (±0)*	−1·3 (±0·15)*

Number of determinations on normal and seizure cats respectively.
Standard deviations given in parentheses after the mean values.

Statistical significance (values not marked were not significantly different from appropriate control.)	0–60 Change	Normal vs. MSO	0–60 Change
	* $P < 0{\cdot}001$ † $P = 0{\cdot}01$	* $P < 0{\cdot}001$ ‡ $P < 0{\cdot}01$ † $P = 0{\cdot}01$	* $P < 0{\cdot}001$ † $P = 0{\cdot}01$

of glutamic acid, glutamine, and γ-aminobutyric acid during incubation for 1 hr *in vitro* (TOWER, 1959*a*). Slice levels of γ-aminobutyric acid, given in Table 1, are somewhat higher than the comparable value of 6·4 μmoles/g found on biopsy of cat frontal cerebral cortex by ROBERTS (1956). It is possible that non-specific interference during fluorimetric assay (LOWE *et al.*, 1958) may account for these differences, but ROBERTS (personal communication) has suggested to us that sampling and preparation of brain slices may lead to higher levels of γ-aminobutyric acid under our conditions, owing to continued functioning of glutamic decarboxylase after tissue sample excision. Data reported elsewhere (TOWER, 1959*b*) are consistent with the latter interpretation. It is quite clear that, whatever the explanation for the higher γ-aminobutyric acid levels may be, the *changes* during incubation are consistent and reproducible, and it is these, rather than the levels themselves, which are important, as is discussed in the text in connexion with Table 2. It can be seen from Table 1 that the free ammonia content of these slices did not change appreciably during incubation. Glutamate (mostly glutamine), which had diffused from slices to incubation media during preparation of samples, was taken up by the slices to a significant extent during 1 hr incubation, as originally demonstrated by STERN *et al.*, (1949). The changes in slice levels during incubation reflect the net results of active turnover of these components of the glutamate system and the restoration of normal levels, disrupted during sampling and preparation, by synthesis from glucose metabolites (TOWER, 1959*a*).

In contrast, the epileptogenic slices exhibited four significant differences. Glutamic acid levels, which were lower than normal initially, continued to decrease during slice incubation. Glutamine was virtually absent from slices initially and, although an increase occurred during incubation, final levels averaged only one-tenth of those in normal slices. This finding is consistent with previous reports that methionine sulphoximine or methionine sulphoxide inhibit glutamine synthesis in various tissue preparations (BOREK *et al.*, 1946; ELLIOTT and GALE, 1948; PACE and MCDERMOTT, 1952; SPECK, 1949), but it is the first demonstration of this effect for incubated brain slices from animals administered methionine sulphoximine *in vivo*. The decrease in levels of γ-aminobutyric acid during incubation of the epileptogenic slices cannot be fully evaluated until more samples have been run. In conformity with the fact that medium glutamate content represents mostly glutamine, the amount present initially in the incubation media of the epileptogenic slices was much lower than that in normal controls, and it essentially disappeared during incubation. The free ammonia content of the epileptogenic slices behaved similarly to that of normal slices.

It is apparent that the administration of methionine sulphoximine *in vivo*, resulting in development of seizures, is associated with profound disturbances of glutamic acid and glutamine metabolism in cerebral cortex samples from these animals, when incubated *in vitro*. The concomitant changes in the electrical activity of the brain *in vivo* are illustrated in Fig. 1, which is an electroencephalographic record from one of these cats, taken before methionine sulphoximine was administered (record A) and during an inter-seizure period after administration and onset of generalized convulsions (record B). Pathological aspects have been discussed elsewhere (HICKS and COY, 1958; TOWER, 1958*a*).

Because of the very low total glutamate (glutamic acid + glutamine) content of these epileptogenic samples, the usual manometric method for determination of total glutamate is operated at the lower limits of its sensitivity. It was considered important

VOL. 5 1959/6

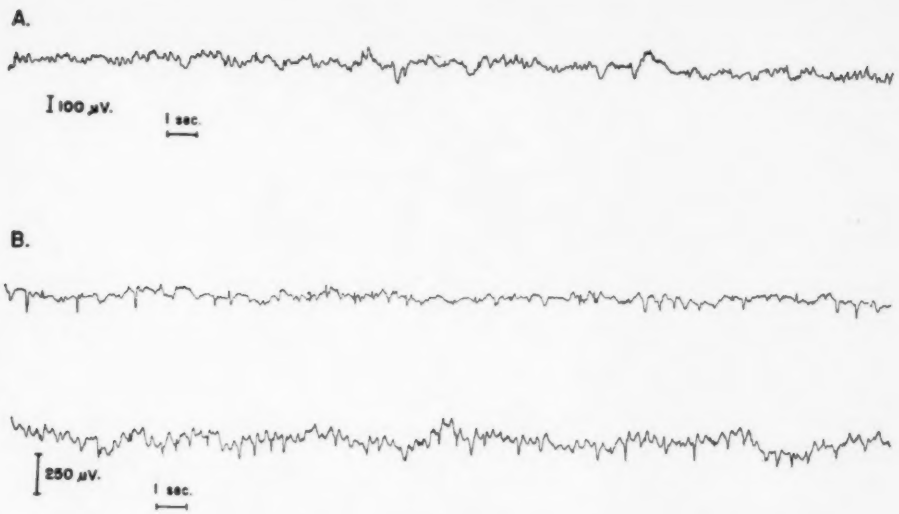


FIG. 1. Electroencephalogram recorded from biparietal skull needle electrodes on an unanaesthetized cat. *Record A*: normal control record. *Record B*: recorded between generalized convulsions after administration of methionine sulphoximine. Note the high voltage slow waves and numerous spikes typical of epileptiform activity. The two lines are continuous. (Time and voltage calibrations given below each record. [The assistance of Dr. Guy Courtois in obtaining these records is gratefully acknowledged.])

to check the validity of the results by a more sensitive method, which permits determination of the other product of bacterial decarboxylation, γ-aminobutyric acid, by fluorimetric means. A comparison of results by the two determinations carried out on the same sample in two normal and two epileptogenic preparations is given in Table 2. The close correspondence of values by the manometric (evolved CO_2) and fluorimetric (γ-aminobutyric acid produced) methods clearly establishes the validity of the results given in Table 1. It is important to point out that variations in slice levels may occur owing to differences in sampling time and techniques and to differences in the state of experimental preparations, as is evident from Table 2. However, the rigidly standardized incubation procedure employed in these studies (TOWER, 1958*b*) permits direct comparison of the *changes* during incubation, regardless of individual slice levels, and it is these changes, reflecting the metabolic capabilities of the slices, which are considered to be the more valid indicators of differences between normal controls and experimental samples.

In vitro *correction of glutamic acid metabolism*. In view of previous findings that toxic effects of methionine sulphoximine could be overcome *in vivo* and *in vitro* by methionine or asparagine (REINER *et al.*, 1950; TOWER, 1959*b*; TOWER and ELLIOTT, 1953), attempts were made to correct the impairments of glutamate metabolism (Table 1) by various *in vitro* additions to the incubating slices. Results are given in Tables 3 and 4. None of the 'effective' compounds, with the possible exception of 2-pyrrolidinone, exhibited any detectable effects on glutamic acid or glutamine metabolism by normal slices (Table 3). The *in vitro* addition of L-asparagine, L-methionine, γ-aminobutyric acid, 2-pyrrolidinone, or adenosine triphosphate (ATP) were all effective in stimulating glutamic acid production by epileptogenic slices, so

TABLE 2.—COMPARISON OF TOTAL GLUTAMATE ANALYSES BY MANOMETRIC AND FLUORIMETRIC PROCEDURES

Preparation	Sample	Total glutamate (glutamic acid + glutamine)			
		Initial content		Content after 1 hr incubation	
		Manometric (μmoles CO_2/g)	Fluorimetric (μmoles γABA/g)	Manometric (μmoles CO_2/g)	Fluorimetric (μmoles γABA/g)
Normal	Tissue slices #1	8·5	8·0	13·8	13·55
	Tissue slices #2	11·5	10·7	15·25	14·9
	Medium for slices #2	4·45	4·5	3·2	3·3
Methionine sulphoximine	Tissue slices #1	7·65	7·55	5·95	—
	+ L-methionine 10 mM	—	—	11·15	11·05
	Tissue slices #2	5·85	5·8	4·9	4·7
	+ L-methionine 10 mM	—	—	8·1	7·8

Analyses based upon reactions catalysed by the glutamic decarboxylase and glutaminase of *Clostridium perfringens* (MEISTER *et al.*, 1951: TOWER, 1958*b*) as follows:

$$\begin{array}{lcl} \text{Glutamic acid} & \rightarrow & \gamma\text{-aminobutryic acid} + CO_2 \\ \text{Glutamine} & \rightarrow & \gamma\text{-aminobutyric acid} + CO_2 + NH_3 \\ & & \downarrow \qquad\qquad\quad \downarrow \qquad\quad \downarrow \\ & & \text{(Fluorimetric analysis)} \quad \text{(Manometric analysis)} \quad \text{(Analysis for glutamine)} \end{array}$$

Each sample was analysed manometrically for CO_2 evolution and the reaction mixture then analysed fluorimetrically for γ-aminobutyric acid (γABA) content, with corrections for reagent, tissue and bacterial 'blanks'. Tissue 'blanks' represented pre-formed or true γ-aminobutyric acid in the samples. The same samples were also analysed for ammonia content (TOWER, 1958*b*) to obtain glutamine content, as amide ammonia.

that after incubation for 1 hr, levels and changes in levels were comparable to those in normal control slices (Table 4). However, none of these compounds affected the inhibition of glutamine metabolism.

Other *in vitro* additions (bottom of Table 4) were ineffective in both cases. The failure of pyridoxal phosphate distinguishes this seizure state from that induced by thiosemicarbazide, in which the derangement of the pyridoxal phosphate-dependent glutamic decarboxylase step by action of the hydrazide is associated with similar abnormalities of glutamic and γ-aminobutyric acid metabolism, but in which, under identical *in vitro* experimental conditions, addition of pyridoxal phosphate definitely corrected these abnormalities (TOWER, 1959*b*). The lack of effectiveness of *n*-valeramide or acetamide (Table 4) suggests that the efficacy of L-asparagine was not due to a non-specific amide effect.

The corrective actions of added methionine or asparagine is consistent with analogous actions under other circumstances cited above. Asparagine may act as a substitute for glutamine (TOWER, 1957*b*) or it may be metabolized by cerebral cortex to aspartic acid and, as the latter, become available for metabolic reactions involving glutamic acid (TOWER, 1959*c*); but neither of these possibilities has been explored in detail. It is not clear from these data why methionine should be effective, but the possibility of several metabolic interrelationships between methionine metabolism

VOL. 5 1959/6

TABLE 3.—GLUTAMATE METABOLISM OF NORMAL CAT CEREBRAL CORTEX SLICES INCUBATED WITH VARIOUS *in vitro* ADDITIONS
(μmoles/g)

Conditions	Incub. time (min)	Glutamic acid		Glutamine	
		Level	Change	Level	Change
Normal controls	60	9·55(±0·9)	+2·15(±0·85)	5·35(±1·0)	+2·2 (±1·0)
+ L-Asparagine 10 mM (2)*	60	9·2 (±0·1)	+1·7	4·55(±0·15)	+2·15
+ L-Methionine 10 mM (1)	60	10·5	+1·85	4·8	+1·6
+ γ-Aminobutyric Acid 10 mM (2)	60	9·6 (±0·85)	+1·65	4·0 (±0)	+1·45
+ 2-Pyrrolidinone 10 mM (1)	60	10·2	+2·25	3·3	+0·75
+ Na-ATP 3 mM (1)	60	8·85	+2·7	5·4	+1·85

* Numbers of determinations. Standard deviations given in parentheses after appropriate values.

and glutamic acid metabolism may be an explanation. Conversion of methionine to cysteine yields α-ketobutyric acid, which might conceivably function as an oxidative substrate. A more likely possibility is the further metabolism of cysteine via cysteine sulphinic acid to pyruvate, an active pathway in cerebral tissue (TOWER, 1957*a*). This series of reactions offers two alternatives—yield of extra pyruvate for conversion via the citric acid cycle to glutamic acid or transamination of cysteine sulphinic acid with α-ketoglutarate to yield glutamic acid directly, with the other product of the reaction, β-sulphinylpyruvic acid, yielding pyruvate, which could be utilized to sustain citric acid cycle operation. The lack of suitably labelled ^{14}C-methionine has prevented further investigation of these possibilities at this time. γ-Aminobutyric acid is intimately related to glutamic acid metabolism and could be functioning in this instance by means of the α-ketoglutarate transamination 'feed-back' (ROBERTS *et al.*, 1958; TOWER, 1959*a*) to build up glutamic acid levels. The efficacy of 2-pyrrolidinone, the lactam of γ-aminobutyric acid, is probably related to the same mechanism, since it has been shown to be converted to γ-aminobutyric acid by cerebral cortex *in vitro* (TOWER, 1957*b* and unpublished). The synthesis and maintenance of glutamic acid in brain is not directly dependent upon ATP, but if cerebral energy metabolism were impaired in these epileptogenic slices, depletion of glutamic acid and γ-aminobutyric acid might have resulted (MCKHANN and TOWER, 1959). The provision of extra energy as ATP might therefore permit repletion of glutamic acid. Several, possibly analogous examples of metabolic effectiveness of *in vitro* addition of ATP have been reported (CALDWELL and KEYNES, 1957; STERN *et al.*, 1949; TOWER, 1956; 1958*b*).

The fact that none of these additions affected the inhibition of glutamine synthesis in these epileptogenic samples is particularly noteworthy. Several of the compounds

TABLE 4.—GLUTAMATE METABOLISM OF CEREBRAL CORTEX SLICES FROM CATS WITH SEIZURES INDUCED BY METHIONINE SULPHOXIMINE INCUBATED WITH VARIOUS *in vitro* ADDITIONS
(μmoles/g)

Conditions	Incub. time (min)	Glutamic acid		Glutamine	
		Level	Change	Level	Change
Methionine Sulphoximine	60	5·5 (±1·05)	−0·95(±0·75)	0·55(±0·35)	+0·35(±0·45)
A. '*Effective*' *compounds*:					
+ L-Asparagine 10 mM (2)	60	8·35(±0·2)*	+1·9	0·0 (±0)	−0·1
+ L-Methionine† 10 mM (5)	60	0·95(±1·35)*	+2·05(±0·95)	0·55(±0·55)	+0·35(±0·45)
+ γ-Aminobutyric Acid 10 mM (3)	60	10·65(±0·7)*	+2·7 (±0·3)	0·35(±0·45)	+0·15(±0·3)
+ 2-Pyrrolidinone 10 mM (2)	60	10·1 (±1·2)*	+2·6	0·45(±0·55)	+0·3
+ Na-ATP 3 mM (2)	60	8·35(±0·7)*	+2·9	0·95(±0·3)	+0·95
B. '*Ineffective*' *compounds*:					
+ Pyridoxal Phosphate 1 mM (1)	60	4·6	−0·5	0·8	+0·8
+ n-Valeramide 10 mM (1)	60	5·45	−0·25	0·95	+0·7
+ Acetamide 10 mM (1)	60	5·15	−0·55	0·9	+0·65

Number of determinations given in parentheses after addition compound. Standard deviations given in parentheses after appropriate values.

* Differ significantly ($P < 0·001$) from unsupplemented controls.

† L-Methionine, 10 mM also produced significant change ($P = 0·01$) in tissue γ-aminobutyric acid level: 8·65 (±1·0) compared to control of 5·3 (±1·6), giving increase during incubation of +2·65 (±1·1) compared with decrease in control of −2·0 (±0·2) in two experiments. Tissue ammonia metabolism on addition of L-methionine, 10 mM was not significantly different from controls (5 experiments): Level: 2·2 (±0·25) vs. 2·5 (±0·4) in controls; change during incubation: +0·4 (±0·2) vs. +0·55 (±0·45) in controls.

effective in correcting glutamic acid metabolism when added *in vitro* are also effective in preventing or arresting the seizures when administered *in vivo*, as well as in correcting certain other abnormalities associated with methionine suphoximine toxicity (inhibition of bacterial growth; impaired 'bound' acetylcholine production by epileptogenic cerebral cortex). If these measures do not restore normal glutamine metabolism, as the data here indicate, it would seem that it is rather their support of glutamic acid metabolism which may be the essential action. As far as epileptogenicity is concerned, evidence from other types of seizure states supports this view (TOWER, 1959*b*). Since ATP is necessary for glutamine synthesis (SPECK, 1949), the addition of ATP here might be expected to stimulate glutamine synthesis, if this were the only factor. The fact that added ATP stimulates glutamic acid synthesis or maintenance but not

VOL. 5 1959/6

glutamine synthesis in these epileptogenic slices strongly indicates some other factor or factors.

In vitro *correction of glutamine metabolism.* Investigations *in vivo* by Du Ruisseau *et al.* (1957) clearly demonstrated that exposure of animals to high levels of circulating ammonia produced striking increases of cerebral glutamine content. These investigators emphasized the role of glutamine synthesis as one of the principal cerebral mechanisms for coping with excess ammonia. The possibility that methionine sulphoximine toxicity involves a disturbance in some facet of cerebral ammonia metabolism was therefore examined.

The effects of adding 10 mM-ammonium chloride (NH_4Cl) to normal cerebral cortex slices during incubation *in vitro* are shown in Table 5. The marked increase in slice

Table 5.—Glutamate metabolism of normal cat cerebral cortex slices incubated in the presence of added ammonia
(μmoles/g)

Conditions	Incub. time (min)	Glutamic acid		Glutamine	
		Level	Change	Level	Change
Normal slices	0	7·45		3·75	
	60	9·6	+2·15	5·1	+1·35
+ NH_4Cl 10 mM	60	5·55	−1·9	9·65	+5·9
	60	6·2	−1·25	9·1	+5·35
Mean		5·85	−1·6	9·4	+5·65
[Net change]*			[−3·75]		[+4·3]

* Change in slices with added NH_4Cl—change in normal slices.

glutamine content at the expense of glutamic acid is comparable to the *in vivo* findings reported by Du Ruisseau *et al.* (1957). Similar experiments were carried out with cerebral cortex slices from cats with seizures induced by methionine sulphoximine. These are summarized in Table 6, together with the data from experiments in which ATP was added. The *in vitro* addition of ATP alone, of L-methionine alone, or of both together had no effect on inhibited glutamine metabolism, despite the fact that any one of these measures definitely corrected impaired glutamic acid metabolism. On the other hand, the *in vitro* addition of 10 mM-NH_4Cl to these epileptogenic slices did result in a significant increase of slice glutamine content, and this effect was considerably enhanced when both NH_4Cl and L-methionine were added. The results in the latter case were analogous to those in Table 5, in the sense that some of the increase in glutamic acid stimulated by addition of methionine alone seemed to be diverted to glutamine synthesis when NH_4Cl was also added. The total glutamate (glutamic acid + glutamine) in these two parallel cases was essentially the same: methionine only —9·8 μmoles/g; methionine + NH_4Cl — 10·1 μmoles/g. It seems reasonable to interpret these results as indicating that stimulation of glutamine synthesis in this situation depended upon adequate production or maintenance of glutamic acid

TABLE 6.—STIMULATION OF GLUTAMINE SYNTHESIS IN INCUBATED SLICES OF CEREBRAL CORTEX FROM CATS WITH SEIZURES INDUCED BY METHIONINE SULPHOXIMINE

Conditions	Incub. time (min)	Glutamic acid				Glutamine			
		Levels		Mean level	Mean change	Levels		Mean level	Mean change
		Exp. 1	Exp. 2			Exp. 1	Exp. 2		
Control	0	5·1	5·8	5·45	—	0·0	0·0	0·0	—
	60	3·25	5·0	4·1	−1·3	1·2	1·0	1·1	+1·1
+ Na-ATP 3 mM	60	8·95	7·75	8·35	+2·9	0·75	1·15	0·95	+0·95
Control	0	7·65	—	—	—	0·0	—	—	—
	60	5·95	—	—	−1·7	0·0	—	—	0·0
+ Na-ATP 3 mM and L-Methionine 10 mM	60	10·65	—	—	+3·0	0·0	—	—	0·0
Control	0	6·2	6·0	6·1	—	0·55	0·95*	0·75	—
	60	5·7	5·2	5·45	−0·65	0·0	0·25*	0·15	−0·6
+ L-Methionine 10 mM	60	9·15	10·45	9·8‡	+3·7	0·0	0·0*	0·0	−0·75
+ NH_4Cl 10 mM	60	6·0	6·3	6·15†	0·05	1·45	1·1*	1·3‡	+0·55
+ NH_4Cl 10 mM and L-Methionine 10 mM	60	6·8	6·9	6·85‡	+0·75	2·95	3·55*	3·25‡	+2·5

* Glutamine data confirmed qualitatively by two dimensional paper chromatography (TOWER, 1958*b*) of duplicate slice samples in this experiment.
† Difference from control significant at $P < 0{\cdot}02$.
‡ Difference from control significant at $P < 0{\cdot}01$.

(induced by the added methionine) plus an excess of ammonia. However, complete restoration to normal (Table 1) was not achieved since the glutamic acid and glutamine levels increased to only about 70 per cent of normal values. The findings are consistent with the suggestion that methionine sulphoximine action may interfere with the normal metabolism of ammonia by cerebral cortex, perhaps by virtue of the imine moiety of the toxic compound.

The data in Tables 5 and 6 point to a remarkable situation in terms of seizure mechanisms. It is well known that administration of excess ammonia can produce seizures and that, associated with this state, there is a marked rise in cerebral glutamine, demonstrable *in vivo* (DU RUISSEAU *et al.*, 1957) and reproducible *in vitro* (Table 5). On the other hand seizures are also produced by methionine sulphoximine, which is associated with inhibition of glutamine synthesis. It does not appear possible to reconcile these two seizure states in terms of a common mechanism involving stimulation of glutamine synthesis by excess ammonia and hence depletion of either citric acid cycle components or glutamic acid for that purpose, as has been suggested (BESSMAN and BESSMAN, 1955). Nor does it appear possible to relate cerebral excitability to a common basis of the ratio of cerebral glutamic acid to glutamine levels, as has also been suggested (WOODBURY, 1958). The possibility that abnormalities of cerebral ammonia in both seizure states might participate in some other common mechanism, such as γ-guanidinobutyric acid production (WAELSCH, personal communication), cannot be ruled out. At the present time the most likely explanation

VOL. 5 1959/6

seems to be a common involvement of glutamic acid and γ-aminobutyric acid metabolism, which is deranged in these seizure states as well as in others where glutamine metabolism is unaffected and ammonia metabolism *per se* seems not to be a factor (TOWER, 1959*b*). The use of methionine sulphoximine as a convulsant agent has proved particularly helpful in focussing critically upon these problems. In addition it has indicated the possibility of a role for methionine in cerebral metabolism not previously suspected and not demonstrable in other seizure preparations (TOWER and ELLIOTT, 1953; TOWER—unpublished).

SUMMARY

The metabolism of glutamic acid and glutamine by incubated slices of cerebral cortex from normal cats and from those with seizures induced by methionine sulphoximine has been compared.

Normal slices exhibited significant increases in glutamic acid and glutamine content, whereas epileptogenic slices exhibited a significant decrease in glutamic acid content and almost complete inhibition of glutamine synthesis.

In vitro addition of L-asparagine, L-methionine, γ-aminobutyric acid, or adenosine triphosphate each effectively stimulated glutamic acid production by the epileptogenic slices without affecting glutamic acid metabolism in normal slices. None of these additions affected glutamine synthesis in epileptogenic slices.

In vitro addition of ammonium chloride alone or in combination with L-methionine stimulated glutamine synthesis in epileptogenic slices to a significant degree.

The possible modes of action of methionine sulphoximine and of the various compounds effective on *in vitro* addition have been discussed and related to general problems of seizure mechanisms.

REFERENCES

BENTLEY H. R., MCDERMOTT E. E. and WHITEHEAD J. K. (1950) *Nature, Lond.* **165,** 735.
BESSMAN S. P. and BESSMAN A. N. (1955) *J. clin. Invest.* **34,** 622.
BOREK E., MILLER H. K., SHEINESS P. and WAELSCH H. (1946) *J. biol. Chem.* **163,** 347.
CALDWELL P. C. and KEYNES R. D. (1957) *J. Physiol.* **137,** 12 P.
DU RUISSEAU J. P., GREENSTEIN J. P., WINITZ M. and BIRNBAUM S. M. (1957) *Arch. Biochem. Biophys.*, **68,** 161
ELLIOTT H. W. and GALE E. F. (1948) *Nature, Lond.* **161,** 129.
HEATHCOTE J. G. and PACE J. (1950) *Nature, Lond.* **166,** 353.
HICKS S. P. and COY M. A. (1958) *A.M.A. Arch. Path.* **65,** 378.
LOWE I. P., ROBINS E. and EYERMAN G. S. (1958) *J. Neurochem.* **3,** 8.
MCKHANN G. M. and TOWER D. B. (1959) *Amer. J. Physiol.* **196,** 36.
MEISTER A., SOBER H. A. and TICE S. V. (1951) *J. biol. Chem.* **189,** 577, 591.
MELLANBY E. (1946) *Brit. Med. J.* **2,** 885.
MELLANBY E. (1947) *Brit. Med. J.* **2,** 288.
MISANI F. and REINER L. (1950) *Arch. Biochem.* **27,** 234.
NEWELL G. W. and CARMAN W. W. (1950) *Fed. Proc.* **9,** 209.
PACE J. and MCDERMOTT E. E. (1952) *Nature, Lond.* **169,** 415.
REINER L., MISANI F. and WEISS P. (1950) *Arch. Biochem.* **25,** 447.
ROBERTS E. (1956) *Progress in Neurobiology* (Edited by KOREY S. R. and NURNBERGER J. I.) Vol. I, p. 11. Hoeber, New York.
ROBERTS E., ROTHSTEIN M. and BAXTER C. F. (1958) *Proc. Soc. exp. Biol., N.Y.* **97,** 796.
RUSSELL J. A. (1944) *J. biol. Chem.* **156,** 457.
SPECK J. F. (1949) *J. biol. Chem.* **179,** 1405.
STERN J. R., EGGLESTON L. V., HEMS R. and KREBS H. A. (1949) *Biochem. J.* **44,** 410.

TOWER D. B. (1956) *Progress in Neurobiology* (Edited by KOREY S. R. and NURNBERGER J. I.) Vol. I, p. 169. Hoeber, New York.
TOWER D. B. (1957*a*) *Ann. Protein Conf.* (*Rutgers*) **13,** 71.
TOWER D. B. (1957*b*) *Clin. Chim. Acta* **2,** 397.
TOWER D. B. (1958*a*) *Temporal Lobe Epilepsy* (Edited by BALDWIN M. and BAILEY P.) p. 288. Thomas, Springfield.
TOWER D. B. (1958*b*) *J. Neurochem.* **3,** 185.
TOWER D. B. (1959*a*) *Biochemistry of the Central Nervous System* (Edited by BRÜCKE F.) *Proc. IVth Int. Congr. Biochem.* Vol. III, p. 213. Pergamon, London.
TOWER D. B. (1959*b*) *Chemical Pathology of the Nervous System* (Edited by FOLCH J.) Pergamon, London, In press.
TOWER D. B. (1959*c*) *Symposium on Neurochemistry of Nucleotides and Amino Acids* (Edited by BRADY R. O. and TOWER, D. B.) Wiley, New York. In press.
TOWER D. B. and ELLIOTT K. A. C. (1953) *J. appl. Physiol.* **5,** 375.
WOODBURY D. M. (1958) *Pharmacol. Rev.* **10,** 275.

VOL.
5
1959/6

Journal of Neurochemistry, 1959, Vol. 5, pp. 91 to 98. Pergamon Press Ltd. Printed in Northern Ireland

THE ISOLATION OF THE COPPER-CONTAINING PROTEIN CEREBROCUPREIN I FROM NORMAL HUMAN BRAIN*

HUNTINGTON PORTER and SALLY AINSWORTH
Department of Neurology, Neurosurgery and Psychiatry, New England Center Hospital, and Department of Medicine (Neurology), Tufts University School of Medicine, Boston, Mass.

(*Received* 6 *March* 1959)

PREVIOUS reports have described the separation from brain tissue under copper-free conditions of three different copper-containing fractions (PORTER and FOLCH, 1957*a*) and the isolation from one of them (bovine fraction I) of cerebrocuprein I, an essentially homogeneous brain protein containing 0·3 per cent copper (PORTER and FOLCH, 1957*b*). Although the copper in the initial preparations of bovine cerebrocuprein I appeared to react directly with sodium diethyldithiocarbamate, bovine cerebrocuprein I prepared by a milder method has subsequently been found to retain more than 90 per cent of its copper in competition with this reagent (PORTER and AINSWORTH, 1958). Since up to one-fourth or more of the pathological copper accumulating in the brains of patients with hepatolenticular degeneration appears to be bound in a form with properties similar to those of fraction I copper-protein(s) from normal human brains (PORTER, 1958), it seemed desirable to isolate cerebrocuprein I from normal human brain as a prerequisite to the determination, by immunological techniques, for example, of the amount of normal cerebrocuprein I present in the brains of patients with hepatolenticular degeneration. The present paper describes the isolation of cerebrocuprein I from normal human brain with further modifications in the isolation procedure.

METHODS AND MATERIAL

Precautions to avoid contamination with extraneous copper, the general conditions of fractionation procedures, criteria for selection of normal human brains for fractionation, the technique of preparative paper electrophoresis on Whatman No. 3MM paper, and the methods used for tissue homogenization, for nitrogen and copper analyses, and for determination of direct-reacting copper were all identical with those employed in previous work (PORTER and FOLCH, 1957*a*, 1957*b*; PORTER and AINSWORTH, 1958). All fractionation procedures were carried out in cold rooms, either at about 4° during manipulation of aqueous solutions or at about −13° during manipulation of aqueous–organic solvent mixtures. All paper electrophoreses were run in copper-free barbital buffer, pH 8·6, ionic strength 0·1 at 250 volts, for 16 hr at 4°. Paper strips were stained with naphthol blue black for protein and the relative optical density at different electrophoretic mobilities was plotted on a Spinco Analytrol recording densitometer. The perforated Teflon plugs used to support the filter paper in conical centrifuge tubes while eluting protein after preparative paper electrophoresis had a screw-threaded central opening into which a threaded nylon rod could be inserted to permit easy removal of the plug from the centrifuge tube without contact with metal. Absorption spectra were measured in a Beckman model DU spectrophotometer with microcuvettes of 3 mm chamber width and 1 cm light path.

* This investigation was supported by research grant B-1733 from the National Institute of Neurological Diseases and Blindness, United States Public Health Service.

Isolation procedure. Two different preparations of cerebrocuprein I from normal human brain have been made with full reproducibility at each step in the isolation procedure. In the preparation described in detail below, 871 gm of whole normal human brain, estimated on the basis of previous analyses of normal human brains (PORTER and FOLCH, 1957*a*) to contain about 4 μg of copper per gram of fresh tissue, were used as the starting material (Table 1). Centrifugation was used throughout to collect insoluble fractions.

TABLE 1.—COPPER CONTENT AND YIELD OF SUCCESSIVE COPPER-RICH FRACTIONS OBTAINED IN THE COURSE OF THE ISOLATION OF HUMAN CEREBROCUPREIN I

Method and step in preparation of fraction	Concentration of copper in fraction† (%)	Yield per 100 g fresh tissue*	
		Solids† (mg)	Copper (μg)
pH 4·5 buffer extract of fresh tissue	0·008	3000‡	238
H_2O extract of first acetone precipitate	0·030	611‡	185
Precipitation with $CHCl_3$–C_2H_5OH	0·095	131	124
Dialysis	0·17§	78	131§
Second acetone precipitation	0·15	53	78
Preparative paper electrophoresis	0·29	5	15

* In calculating yields of copper and of protein per 100 g fresh tissue, the aliquots of copper-rich fractions utilized in pilot experiments or otherwise subtracted during the fractionation procedure have been taken into account.

† Based on dry weight.

‡ Buffer extracts of the fresh tissue contained much dialysable material, some of which was carried down in the first acetone precipitation. Dialysed buffer extracts of the fresh tissue contained about 600 mg of protein per 100 g of fresh tissue.

§ The dialysed material appeared to contain two types of copper–protein combination: (a) cerebrocuprein I; (b) a colourless copper-binding protein with a more rapid electrophoretic mobility than cerebrocuprein I. The bulk of the copper in this second form was removed in the subsequent acetone precipitation step.

Fraction I, previously found (PORTER and FOLCH, 1957*a*) to contain an average of 2·52 μg of copper per gram of normal human brain or about 60 per cent of the total tissue copper in both human cortex and human white matter, was prepared by extracting the homogenized fresh tissue with 0·1 M-sodium acetate buffer, pH 4·5 (4 ml buffer per gram fresh tissue). To the supernatant fluid (fraction I) 3 volumes of precooled acetone were added at $-13°$ and the mixture was allowed to stand for 16 hr. The bulk of the clear supernatant fluid was siphoned off and discarded, the precipitate which had settled was collected, and the bulk of the residual acetone remaining with the precipitate was allowed to evaporate by standing the centrifuge tubes on their sides at $-13°$ for 24 hr.

Sufficient water, redistilled over glass, was added to the precipitate to make a suspension having a volume 1/10 the decanted acetate buffer extract from which the precipitate had been obtained. The suspension was stirred for 30 min and the insoluble material discarded. The supernatant was allowed to stand at $-13°$ until some ice crystals formed. Precooled chloroform–ethanol mixture (1 : 14) (MANN and KEILIN, 1938; MARKOWITZ, CARTWRIGHT and WINTROBE, 1959) was then added slowly, with mechanical stirring, to a concentration of 45% (v/v). The mixture was allowed to stand for 10 min and the resulting large precipitate (containing only 0.009% Cu) was discarded. To the chloroform-ethanol supernatant was added acetate buffer pH 5·2 ionic strength 0·2, to a volume 1/10 that of the starting aqueous supernatant fluid. Precooled ethanol (without chloroform) was then added to a final concentration of 75% (v/v) chloroform–ethanol, and the mixture was allowed to stand for 90 min. The resulting pale pink precipitate was collected and the bulk of the residual organic solvents was allowed to evaporate overnight as before.

To the precipitate was added sufficient distilled water to make a suspension containing about

VOL. 5 1959/6

2·5% solutes (as estimated by dry weight of the precipitate obtained from an aliquot of the supernatant fluid of 45% chloroform–ethanol) and the suspension was dialysed against 133 volumes of distilled water for 40 hr with constant mechanical shaking. The small amount of insoluble material remaining was discarded, and to the supernatant fluid, now containing about 0·7% solutes, was added 1/10th its volume of sodium acetate buffer, pH 5·2, ionic strength 0·2. An equal volume of precooled acetone was added and the mixture was allowed to stand for 2 hr. The resulting precipitate was collected and the bulk of the residual acetone was allowed to evaporate as above. (The precipitate obtained by adding an equal volume of acetone to the supernatant fluid from the above step, i.e. by raising the acetone concentration from 50 to 75% (v/v), contained 0·28% copper. However, most of the copper protein contained in this subfraction differed from cerebrocuprein I in being colourless and in showing an electrophoretic mobility similar to that of plasma albumin. This subfraction was therefore discarded from the present preparation. The possible significance of the copper in this subfraction is commented upon in the discussion.)

The material precipitated at 50% (v/v) acetone concentration, estimated to have a dry weight of 268 mg, was dissolved in sufficient 0·2 M-ammonium bicarbonate to give a volume of 1·8 ml and fractionated by preparative paper electrophoresis. 0·4 ml of the solution was placed on each of 4 strips of Whatman No. 3MM filter paper, each 6 cm wide, and the electrophoresis of the material on the 4 strips was carried out simultaneously. As in the case of material from bovine brain, two coloured bands, one green and the other pink, were visible on the unstained strips after completion of the electrophoresis. The strips were cut into bands of from 1 to 2 cm in length, the first band cut from each strip being about 1 cm long, and centred at the zone of maximal green colour. Bands of corresponding mobility were pooled and their protein was eluted by centrifugation followed by washing the papers in each tube 3 times with barbital buffer as previously described (PORTER and FOLCH, 1957*b*). The eluate from the pooled bands of maximal green colour contained human cerebrocuprein I with properties similar in general to those of the previously described copper–protein isolated from bovine brain (PORTER and FOLCH, 1957*b*). In the tube containing this fraction, care was taken not to mix the original concentrated eluate at the bottom of the tube with the more dilute washings which remained layered above it until aliquots of the original concentrated eluate had been taken for analytical paper electrophoresis. This procedure seemed less cumbersome and less likely to introduce contamination with extraneous copper than the alternative of attempting the transfer under copper-free conditions of the 4 paper bands pooled in this tube to a second plug-fitted centrifuge tube for washing. After the aliquots for analytical paper electrophoresis had been taken the original eluate and washings were mixed and aliquots were taken for (1) the determination of direct-reacting and total copper, (2) dialysis against redistilled water prior to the determination of dry weight, nitrogen, and copper, and (3) dialysis against copper-free 0·15 M-NaCl prior to ultracentrifugation and measurement of absorption spectrum. The sum total of copper in all the eluted fractions was about 80 per cent of the copper in the material placed on the paper. The material eluted from the pooled bands of maximal green colour amounted to about 22 mg and accounted for about 25 per cent of the total eluted copper.

PROPERTIES OF HUMAN CEREBROCUPREIN I

Homogeneity and molecular weight. Ultracentrifugation analyses, carried out by Dr. KARL SCHMID of the Massachusetts General Hospital, showed a sedimentation coefficient of $S_{20,w} = 3{\cdot}1\ S$, indicating a molecular weight of the order of 30,000 to 40,000.* Ultracentrifugation photographs (Fig. 1) indicated that the preparation was at least 85 per cent homogeneous. Quantitative paper electrophoresis (Fig. 2) indicated a similar degree of homogeneity and showed the protein to have an electrophoretic mobility most nearly approximating, but slightly less than, that of a plasma α_2 globulin.

Copper and nitrogen content. Based on dry weight, the preparation contained 0·29 per cent copper, which, with the sedimentation coefficient given, would indicate 2 atoms of copper per molecule. It is probable that small amounts of contaminants

* A partial specific volume of the order of 0·72 ml g being assumed.

containing no copper were present in this preparation and that the actual copper content of the naturally occurring copper-protein is slightly higher. The nitrogen content of this preparation was 15·7 per cent.

Absorption spectrum. The original concentrated eluate after preparative paper

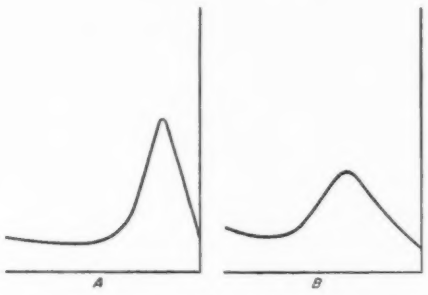


FIG. 1.—Tracings of ultracentrifugation photographs of human cerebrocuprein I. Curve A after 59 min ultracentrifugation; curve B after 123 min ultracentrifugation. Protein concentration was about 0·5 per cent.

VOL. 5 1959/6

electrophoresis, before dilution with the washings from the papers, was a deep bluish green. In the absorption spectrum, illustrated in Fig. 3, the extinctions ($E^{1\%}_{1cm}$) shown are based on nitrogen analyses of samples of the diluted solutions in the spectrophotometer cuvettes after completion of the spectrum. The absolute values of the

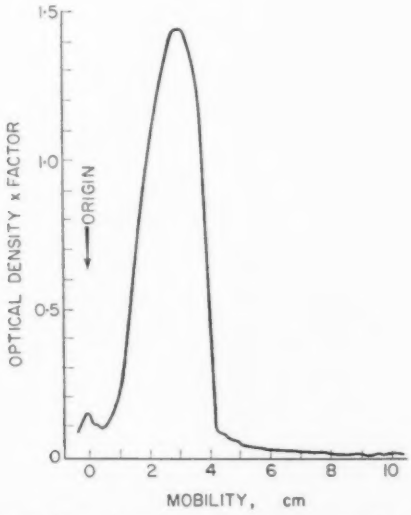


FIG. 2.—Quantitative paper electrophoresis of human cerebrocuprein I on Whatman No. 1 paper. Protein concentration was about 3 per cent.

illustrated relations of absorption to protein concentration must be considered as only approximate. The absorption spectrum of human cerebrocuprein I is similar to that observed in the case of material from bovine brain (PORTER and FOLCH, 1957*b*) in showing a plateau between 600 and 700 mμ with a maximum at 665 mμ and in showing more absorption at 270 mμ than at 280 mμ with a relatively high ratio of

absorption at 260 mμ to absorption at 280 mμ. As discussed (PORTER and FOLCH, 1957*b*) with reference to one preparation of bovine cerebrocuprein I, the peak at 405 mμ may be attributed to the presence of small amounts of contaminants similar to those separating to form the pink band in the preparative paper electrophoresis step.

Stability of the copper–protein bond. The capacity of the copper in purified human cerebrocuprein I to react directly with sodium diethyldithiocarbamate was determined by the method previously described (PORTER and AINSWORTH, 1958). The total copper content of the samples analysed was confirmed by analysis for total copper of the

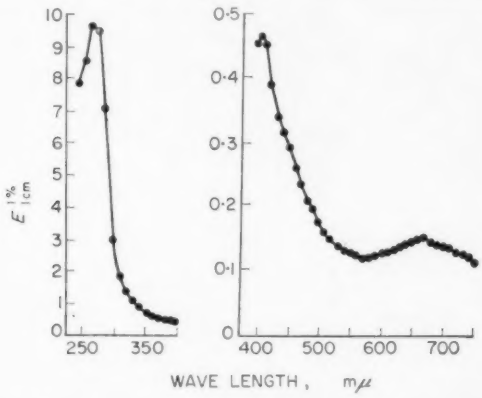


FIG. 3.—Absorption spectrum of human cerebrocuprein I. Protein concentrations at the time of measurement were approximately 0·5 per cent for the visible range and 0·2 per cent for the ultraviolet.

material in the spectrophotometer cuvettes after completion of the test for direct reaction. In two different preparations of the copper protein, aliquots of the eluate from preparative paper electrophoresis containing respectively 5·25 μg and 6·39 μg of total copper were analysed. The direct-reacting copper amounted to 0·27 μg and 0·18 μg, or 5 per cent and 3 per cent respectively, of the total copper in the samples.

The stability of the copper–protein bond at different pH's has been measured both in total fraction I extracts of the fresh tissue and in human cerebrocuprein I purified to the second acetone precipitation stage with a copper content of 0·15 per cent. The latter retained more than 95 per cent of its copper on dialysis at pH 4·5 and more than 85 per cent of its copper on dialysis at pH 4·0. The total fraction I copper in pH 8·2, pH 5·2 and pH 4·5 buffer extracts of normal human brain was also undialysable at the pH's of the extracts. The dialysability of normal human fraction I copper below pH 4·5 was investigated in the following manner. Aliquots of pH 4·5 acetate buffer extract corresponding to about 7 g of fresh tissue were dialysed for 24 hr against 25 volumes of distilled water and then against single 25 volume portions of acetate or citrate buffers of ionic strength 0·1 for 72 hr with frequent shaking during each day. At the end of this period the copper in the external as well as the internal solutions was measured, and the copper bound at each pH was calculated according to the formula (bound copper concentration) equals (total copper concentration inside dialysis bag) minus (copper concentration outside dialysis bag). The amounts of copper bound at different pH's, expressed as percentage of the copper present in the original extract before dialysis, are shown in Fig. 4.

DISCUSSION

Human cerebrocuprein I as isolated here was similar to bovine cerebrocuprein I (PORTER and FOLCH, 1957*b*) in having a copper content of 0·3 per cent and a molecular weight estimated to be of the order of 30,000 to 40,000, in its green colour with an absorption maximum at 665 mμ, in its ability to retain its copper on dialysis at a pH as acid as 4·5, and in the failure of its copper to react directly with sodium diethyldithiocarbamate (PORTER and AINSWORTH, 1958). It appeared to differ from the bovine copper–protein in having a slightly greater electrophoretic mobility at pH 8·6

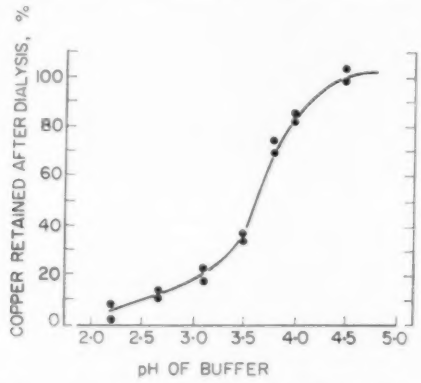


FIG. 4.—Effect of pH on the dialysability of human fraction I copper. Copper retained expressed as percentage of the copper present in the original extract of the fresh tissue before dialysis. Dialysis at pH 3·8 and above against sodium acetate buffers. Dialysis at pH 3·5 and below against citrate–NaOH–HCl buffers.

and in having a spectrum showing somewhat more absorption in the 260–280 mμ range. Whether or not these differences will be confirmed in subsequent preparations and are a reflection of chemical differences such as differences in amino acid composition between the human and bovine proteins has not yet been determined. The properties of cerebrocuprein I which suggest that the copper may be bound to the protein through carboxyl groups have been commented upon in a previous report (PORTER and FOLCH, 1957*b*).

Cerebrocuprein I has some properties, for example, its absorption spectrum showing a maximum of low extinction at about 660 mμ and its ability to retain all its copper on dialysis at a pH as acid as 4·5, which had not been previously described in such copper–proteins as hemocuprein and hepatocuprein (MANN and KEILIN, 1938), ceruloplasmin (HOLMBERG and LAURELL, 1948, 1951), and a copper–protein from horse liver (MOHAMED and GREENBERG, 1954). Since publication of the original report on the isolation of bovine cerebrocuprein I (PORTER and FOLCH, 1957*b*), the isolation of erythrocuprein from human erythrocytes has been described (MARKOWITZ, CARTWRIGHT and WINTROBE, 1959; KIMMEL, MARKOWITZ and BROWN, 1959). Properties of erythrocuprein which are similar to those of cerebrocuprein I include a sedimentation coefficient of the order of $S_{20,w} = 3{\cdot}0\ S$, bluish green colour with an absorption spectrum showing a maximum at about 660 mμ but relatively low extinction at this wave length and showing more absorption at 270 mμ than at 280 mμ, ability to retain copper in competition with carbamate and at a pH as acid as 4·5, and selective separation at pH 5·2 during isolation procedures. It is, therefore, possible that

VOL.
5
1959/6

erythrocuprein may prove to have a structure and function similar to those of cerebrocuprein I.

During several attempts to isolate cerebrocuprein I from normal human brain it has been consistently found that, in some steps, subfractions having the greatest non-dialysable copper content did not contain the largest amounts of cerebrocuprein I; these results indicate that at least one other type of copper–protein combination was present. Thus, in the present method, the subfraction precipitating between 50% and 75% (v/v) acetone in the second acetone precipitation step contained 0·28 per cent copper, but on preparative paper electrophoresis of this subfraction more than 70 per cent of the eluted copper was recovered in a colourless form at a mobility similar to that of plasma albumin. Treatment of the material from pH 4·5 extracts of normal human brain with chloroform–methanol (2:1) at room temperature, as is done in the isolation of bovine cerebrocuprein I (PORTER and FOLCH, 1957*b*), or with chloroform–ethanol (1:14) at about 0°, under conditions approximating those employed in the isolation of erythrocuprein (MARKOWITZ, CARTWRIGHT and WINTROBE, 1959),* yielded larger amounts of such colourless rapidly moving copper–protein combinations. Although the copper–protein combinations of this type obtained in the course of the present preparations have not been studied more extensively, they may be similar to a copper-binding protein isolated from pH 8·2 extracts of normal human brain (PORTER and FOLCH, 1957*c*; PORTER, 1958) which was also colourless and showed an electrophoretic mobility at pH 8·6 similar to that of plasma albumin. This protein was found to contain up to 1 per cent copper in a form not dialysable at pH 4·5 and to have a molecular weight of the order of only 10,000. The copper in this protein, and in the subfractions with similar electrophoretic mobility obtained during the course of the present method, seems unlikely to represent solely contamination with extraneous copper in view of the failure of the copper to react directly with carbamate, whereas 99 per cent of copper added to brain proteins by dialysis reacted directly (PORTER and AINSWORTH, 1958). In view of their very high copper content and their variability in yield, copper–protein combinations of the above type are considered probably to represent artefacts, possibly owing to protein breakdown either in the immediate post-mortem period or during the isolation procedure. The amount of cerebrocuprein I isolated by the present method accounts for only about 6 per cent of the total copper in normal human fraction I. Further investigation will be required to determine whether or not fraction I from normal human brain, which accounts for about 60 per cent of the total brain copper (PORTER and FOLCH, 1957*a*), contains other naturally occurring copper–proteins.

SUMMARY

The copper-containing protein cerebrocuprein I has been isolated from normal human brain. Human cerebrocuprein I is similar to the previously isolated bovine cerebrocuprein I in having a copper content of 0·3 per cent and a sedimentation coefficient of 3·1 *S*, in its green colour with a broad absorption maximum of low extinction at 665 mμ, in its ability to retain its copper on dialysis at a pH as acid as 4·5, and in the failure of its copper to react directly with sodium diethyldithiocarbamate. It appears to differ from bovine cerebrocuprein I in having a slightly greater

* Dr. G. E. CARTWRIGHT very kindly made available details concerning the isolation and properties of erythrocuprein prior to the publication of this reference.

electrophoretic mobility at pH 8·6 and in having a spectrum showing greater absorption in the 260–280 mμ range.

Acknowledgement—The helpful advice of Dr. JORDI FOLCH in the preparation of this manuscript is gratefully acknowledged.

REFERENCES

HOLMBERG C. G. and LAURELL C. B. (1948) *Acta chem. scand.* **2,** 550.
HOLMBERG C. G. and LAURELL C. B. (1951) *Acta chem. scand.* **5,** 476.
KIMMEL J. R., MARKOWITZ H. and BROWN D. M. (1959) *J. biol. Chem.* **234,** 46.
MANN T. and KEILIN D. (1938) *Proc. Roy. Soc.* B **126,** 303.
MARKOWITZ H., CARTWRIGHT G. E. and WINTROBE M. M. (1959) *J. biol. Chem.* **234,** 40.
MOHAMED M. S. and GREENBERG D. M. (1954) *J. gen. Physiol.* **37,** 433.
PORTER H. (1958) In *Chemical Pathology of the Nervous System; 3rd Int. Symp. of Neurochemistry* (Edited by FOLCH J.) Pergamon Press, London. In press.
PORTER H. and AINSWORTH S. (1958) *Proc. Soc. exp. Biol., N.Y.* **98,** 277.
PORTER H. and FOLCH J. (1957*a*) *Arch. Neurol. Psychiat., Chicago* **77,** 8.
PORTER H. and FOLCH J. (1957*b*) *J. Neurochem.* **1,** 260.
PORTER H. and FOLCH J. (1957*c*) *Proc. 1st. Int. Congr. on Neurological Sciences* (Edited by VAN BOGAERT L.) Stechert-Hafner, New York, In press.

VOL.
5
1959/6

Journal of Neurochemistry, 1959, Vol. 5, pp. 99 to 100. Pergamon Press Ltd. Printed in Northern Ireland

PRELIMINARY NOTE

Reversal of the reserpine-induced depletion of brain serotonin by a monoamine oxidase inhibitor

(*Received* 2 *June* 1959)

OL.
5
9/60

It has been demonstrated that iproniazid (IPN) is not able to restore serotonin and norepinephrine levels after they have been depleted from the brain by reserpine treatment (Brodie and Shore, 1957). This finding agrees with the hypothesis of the relative inefficacy of IPN in inhibiting monoamine oxidase (MO) *in vivo* (Udenfriend, Weissbach and Bogdanski, 1957). On the other hand a stronger inhibitor of MO, phenylisopropylhydrazine (Horita, 1958), induces a marked increase of brain serotonin after depletion by reserpine (Brodie, Spector, Kuntzman and Shore, 1958); this supports the view that reserpine does not affect the biosynthesis of serotonin.

Table 1.—Effect of IPN and IPH injection on the serotonin content of the rat brain

The drugs were injected intraperitoneally 6 hr before killing. The number of rats is given in brackets.

Treatment	Brain serotonin (μg/g wet weigh) $\pm$ S.E.M.
Controls (15)	0·470 $\pm$ 0·021
IPN 100 mg/kg (10)	0·994 $\pm$ 0·038
IPH 61 mg/kg (10)	1·860 $\pm$ 0·065

The present study with isopropylhydrazine (IPH), a MO inhibitor more active than IPN (Pletscher and Gey, 1958) and free from the central excitatory effects of phenylisopropylhydrazine (Biel, 1958), has been undertaken in order to demonstrate (a) an effect on brain serotonin levels after reserpine-induced depletion and (b) an interrelationship between serotonin levels in brain and reserpine sedation. Albino rats weighing about 150 g were used. Reserpine (Serpasil CIBA), 5 mg/kg, was administered subcutaneously 18 hours before killing. Serotonin was determined in the whole brain by the spectrophotofluorimetric technique (Bogdanski, Pletscher, Brodie and Udenfriend, 1956).

The effects of the drugs on brain serotonin concentration are compared in Table 1. IPH is much more effective than IPN in increasing brain serotonin levels. Changes in brain serotonin induced by IPN and IPH after depletion by reserpine are reported in Table 2. Little effect is shown by IPN, while IPH restores the serotonin content to normal values. Moreover we may assume that, owing to the pretreatment with reserpine, the serotonin is present in this last condition exclusively in the *free form.*

Although IPN reverses the depletion of brain serotonin induced by reserpine, the pharmacological effects of reserpine on the central nervous system are still present, as demonstrated by the hypothermia, sedation, ptosis and blocking of a previously established avoidance-escape conditioned reflex. The sedative action of reserpine is therefore present even when the concentration of brain serotonin is high.

The norepinephrine concentration in the brain was not determined by us under these conditions,

but it is known (SPECTOR, PROCKOP, SHORE and BRODIE, 1958; SHORE, 1958) that the increase of norepinephrine after MO inhibition is slower and less marked than that of serotonin. We may suppose that in our experiments the rats with high concentrations of free brain serotonin still had very low norepinephrine levels. If this last point could be demonstrated, we would be able to state

TABLE 2.—EFFECT OF IPN AND IPH ON THE SEROTONIN CONTENT OF THE RAT BRAIN AFTER RESERPINE DEPLETION

Reserpine was injected subcutaneously 18 hr before, and MO inhibitors intraperitoneally 6 hr before killing. The number of rats is given in brackets.

Treatment	Brain serotonin (μg/g wet weigh) $\pm$ S.E.M.
Reserpine 5 mg/kg (6)	0·072 $\pm$ 0·002
Reserpine 5 mg + IPN 100 mg/kg (6)	0·152 $\pm$ 0·027
Reserpine 5 mg + IPH 61 mg/kg (10)	0·460 $\pm$ 0·045

in agreement with BRODIE, BOGDANSKI and SHORE (1958), that the sedative action of reserpine still persists owing to the prevalence of the serotonin-dependant over the norepinephrine-dependant systems.

N. CANAL
A. MAFFEI-FACCIOLI

Institute of Pharmacology of the University
Milan

REFERENCES

BIEL J. H., NUHFER P. A. and CONWAY A. C. (1959) *Ann. N. Y. Acad. Sci.* **80,** 568.
BOGDANSKI D. F., PLETSCHER A., BRODIE B. B. and UDENFRIEND S. (1956) *J. Pharmacol.* **117,** 82.
BRODIE B. B., BOGDANSKI D. F. and SHORE P. A. (1958) *Chemical Concepts of Psychosis* (Edited by RINKEL M. and DENBER H. C. B.), p. 190, McDowell, New York.
BRODIE B. B. and SHORE P. A. (1957) *Ann. N.Y. Acad. Sci.* **66,** 631.
BRODIE B. B., SPECTOR S., KUNTZMAN R. and SHORE P. A. (1958) *Naturwissenschaften* **45,** 243.
HORITA A. (1958) *J. Pharmacol.* **122,** 176.
PLETSCHER A. and GEY K. F. (1958) *Helv. physiol. acta* **16,** 26 C.
SHORE P. A. (1958) *J. clin. exp. Psycopath.* **19,** 56.
SPECTOR S., PROCKOP D., SHORE P. A. and BRODIE B. B. (1958) *Science* **127,** 704.
UDENFRIEND S., WEISSBACH H. and BOGDANSKI D. F. (1957) *J. Pharmacol.* **120,** 225.

VOL.
5
1959/6

Journal of Neurochemistry, 1959, Vol. 5, pp. 101–102. Pergamon Press Ltd. Printed in Northern Ireland

ANNOUNCEMENTS

National Hospital Centenary

THE National Hospital, Queen Square, will be celebrating its centenary in 1960 during the period 20th June through 25th June. The occasion will be marked by neuro-historical presentations, as well as by a wide range of social functions. Personal invitations are in the course of being sent to every former student and house officer at the hospital. In addition, the hospital will be extending a most cordial welcome to neurologists, neurosurgeons and others, to participate in these activities. An *ad hoc* notice will be forwarded, not only to individuals who have worked at Queen Square, but to all official neurological and neurosurgical Societies. For details, apply to Dr. Macdonald Critchley, National Hospital, Queen Square, London, W.C.1.

OL.
5
9/60

World Federation of Neurology

THE Commission of Neurochemistry of the World Federation of Neurology met for the first time at the Institut Bunge in Antwerp, Belgium on September 29 and 30, 1959. Dr. Ludo van Bogaert, President of the Federation, and Dr. Charles Poser, Medical Executive Officer, assisted at the meeting.

The following neurochemists had been invited and constituted the original Commission:

Dr. BAUER	Hamburg, Germany
Dr. BRANTE	Eskilstuna, Sweden
Dr. CUMINGS	London, England
Dr. EDGAR	Amsterdam, Netherlands
Dr. FOLCH-PI	Waverley, Mass., U.S.A.
Dr. KOREY	New York, U.S.A.
Dr. LOWENTHAL	Antwerp, Belgium
Dr. PEARSE	London, England
Dr. QUASTEL	Montreal, Canada
Dr. RICHTER	Cardiff, England
Dr. SVENNERHOLM	Göteborg, Sweden
Dr. TOURTELOTTE	Ann Arbor, Mich., U.S.A.

The Commission discussed the participation of neurochemists in the different international congresses. A permanent secretariat is to be established at the Institut Bunge, in Antwerp. Dr. A. Lowenthal was named Secretary of the Commission.

Plans were laid for holding a Symposium on Neurochemistry in Rome during the Congress of Neurology of 1961. The two major topics to be covered are: (1) Lipids, lipoproteins and their metabolism; (2) Disorders of myelin (not including multiple sclerosis).

The Commission plans to establish a Registry of neurological material available for neurochemical investigation. In the near future, it is hoped that neurological associations and neuropathological centres will be informed of the type of material wanted by the neurochemists and under what conditions this material is to be obtained and transported. All requests and inquiries should be addressed to the Secretary of the Commission, who will see to it that the necessary contacts are made between physicians offering material and those requesting it for chemical study. It should be understood that although collaborative efforts are encouraged, each investigator retains full publication rights concerning the material he furnishes.

The Secretary of the Commission will endeavour to keep the various medical and scientific journals informed about the activities of the Commission and welcomes any suggestions from interested physicians. Enquiries should be sent to Dr. A. LOWENTHAL, Institut Bunge, 59, rue Philippe Williot, Berchem-Anvers, Belgium.

VOL. 5 1959/6

Drugs Affecting Lipid Metabolism

AN international Symposium will be held in Milan, Italy, on June 2–4 1960, for the purpose of reviewing the present status of biological and clinical research on drugs affecting lipid metabolism. The agenda will include four main subjects:

(1) New data on cholesterol and lipid metabolism (biosynthesis, absorption, site, catabolism, excretion, abnormal pathways).
(2) Experimental methods for the evaluation of drugs affecting cholesterol and lipid metabolism (new analytical methods and pharmacological tests).
(3) Drugs affecting cholesterol and lipid metabolism in relation to the prevention and treatment of experimental atherosclerosis.
(4) Clinical methods and therapeutical significance of drugs affecting cholesterol and lipid metabolism.

The Symposium, sponsored by the Institute of Pharmacology of the University of Milan, will be under the Chairmanship of Prof. E. TRABUCCHI. Information on participation and presentation of papers can be obtained from Prof. S. GARATTINI, c/o Institute of Pharmacology, Via A. del Sarto 21, Milan, Italy, until 1 March.

The official languages are Italian and English. Simultaneous translation will be available.

The registration fee is $10.

Further announcements will inform about the detailed programme.

Journal of Neurochemistry, 1959, Vol. 5, p. 103. Pergamon Press Ltd. Printed in Northern Ireland

Papers to be published in future issues

D. M. ROBERTSON: The paper electrophoretic distribution of soluble proteins in different regions of the human brain.

M. WENDER and M. HIEROWSKI: The concentration of electrolytes in the developing nervous system with special reference to the period of myelination.

J. KRAWCZYŃSKI, ST. WISZNIOWSKA and I. DREWNOWSKA: Incorporation of radioactivity into proteins of the cerebrum, cerebellum and other organs, after the injection of ^{35}S in the form of sodium sulphate.

H. H. HESS and A. POPE: Intralaminar distribution of cytochrome oxidase activity in human frontal isocortex.

C. S. SPYROPOULOS: Cytoplasmic pH of nerve fibres.

A. F. HARRIS and A. SAIFER: The metachromasy of strandin.

M. H. APRISON, A. LUKENBILL and W. E. SEGAR: Sodium potassium, chloride and water content of six discrete parts of the mammalian brain.

A. BONICHON: Développement de la cholinestérase au niveau du mésencéphale chez l'embryon de poulet.

C. F. BAXTER, E. ROBERTS and E. EIDELBERG: γ-Aminobutyric acid and seizure susceptibility in areas of normal cat brain cortex.

N. M. SULKIN: The distribution of mucopolysaccharides in the cytoplasm of vertebrate cells.

C. F. EMANUEL and I. L. CHAIKOFF: Desoxyribonucleic acids of central nervous system, kidney and spleen: a comparison of some chemical and physical properties.

R. KATO: Serotonin content of rat brain in relation to sex and age.

G. GÁRDOS: Potassium accumulation in guinea pig brain cortex slices.

R. L. FRIEDE: Histochemical investigations on succinic dehydrogenase in the central nervous system—IV. A histochemical mapping of the cerebral cortex of the guinea pig.

S. ROSENBLATT, J. CHANLEY, H. SOBOTKA and M. R. KAUFMAN: Interrelationships between electroshock, the blood-brain barrier and catecholamines.

A. J. HUDSON, J. H. QUASTEL and P. G. SCHOLEFIELD: The effects of heated snake venom on the phosphate metabolism of the rat spinal chord.

M. WOLMAN and S. HESTRIN-LERNER: A histochemical contribution to the study of the molecular morphology of the myelin sheath.

W. G. VAN DER KLOOT: Factor S—a substance which excites crustacean muscle.

M. J. E. ERNSTING, W. F. KAFOE, W. T. NAUTA, H. K. OOSTERHUIS and C. DE WAART: Biochemical studies on psychotropic drugs—I. The effect of psychotropic drugs on γ-aminobutyric acid and glutamic acid in brain tissue.

B. MCARDLE, R. H. S. THOMPSON and G. R. WEBSTER: The action of lysolecithin and of snake venom on whole-cell preparations of brain, muscle and liver.

L. H. COHEN and W. K. NOELL: Glucose catabolism of rabbit retina before and after development of visual function

J MAGNES and S. HESTRIN-LERNER: Effect of 5-hydroxytryptophan and serotonin on glucose content and functional activity of the perfused cat brain.

K. YAGI, T. OZAWA, M. ANDO and T. NAGATSU: The effect of flavin adenine dinucleotide on the EEG. modified by chlorpromazine.

G. PORCELLATI and B. CURTI: Proteinase activity in peripheral nerves during Wallerian degeneration.

M. KUROKAWA: Metabolic consequences of localized application of electrical pulses to sections of the cerebral white matter.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 105–108. Pergamon Press Ltd. Printed in Northern Ireland

THE CONCENTRATION OF ELECTROLYTES IN THE DEVELOPING NERVOUS SYSTEM WITH SPECIAL REFERENCE TO THE PERIOD OF MYELINATION

MIECZYSLAW WENDER and MARIAN HIEROWSKI
Neurological Clinic and Institute of Physiological Chemistry,
Medical Academy, Poznań, Poland

(*Received* 26 *February* 1959)

OL.
5
9/60

INVESTIGATIONS of the chemical composition of developing nervous tissue aim at correlating changes in the concentrations of chemical compounds with the differentiation of this tissue. Many of these investigations, especially those concerning the period of myelination, have been carried out with reference to cerebral lipids. Data on the developmental changes of inorganic constituents are few and rather incomplete. Investigations on developmental changes in white matter, which consists chiefly of myelinated nerve fibres and oligodendroglia, are of particular importance when made in conjunction with histological examination of the stage of myelination, one of the basic processes in the functional maturation of nerve fibres. The results of these studies not only supply information on the chemical changes during myelination but may also yield indirect evidence regarding the chemical composition of the myelin sheath. The developmental changes occurring in grey matter and in the spinal cord are more difficult to interpret. The development of grey matter involves not only the maturation of the nerve and glia cells, but also the myelination of varying numbers of nerve fibres present in the grey matter in different parts.

The present report contains the results of investigations on changes in the content of sodium, potassium and calcium during the development of the brain and spinal cord of the guinea pig, consideration being also given to changes in the water content. From SPERRY's (1955) survey on the biochemistry of the brain during early development, which contains only scanty data on this subject, it appears that there occurs a decrease in the content of sodium, potassium and calcium in many animals after birth. In the guinea pig, however, the level of calcium shows an increase during this period of life. In previous investigations these changes have not been compared with the process of myelination.

METHODS

Investigations were made on a total of 70 guinea pigs of both sexes which were divided into 7 age-groups with 10 animals in each group: foetuses in the 8–9th week of uterine life, animals at the age of 3, 5, 9 and 12 days, 2 months and 6–8 months.

The animals were anaesthetized with ether and killed by cutting the heart; the brain and spinal cord were removed as soon as possible. Samples of grey matter were taken from all cerebral lobes by cutting off the cortex. The portions of white matter for chemical examination included the white matter of the cerebral hemispheres, the corpus calosum, the internal capsule and the macroscopically visible parts of white matter in the cerebral trunc and the cerebellum. Samples of foetal white matter were taken from those topographical areas in which it appears after birth. The samples were

immediately weighed and lyophilized. Separate small slices were fixed in 15% (w/v) formalin for histological examination. The lyophilized material was weighed to constant weight after drying. From these values the water content of the tissue was calculated. The dry residue was heated for 1 hr at about 450° and then extracted for 24 hr with 0·5 N-HCl at 18°. The extract was then filtered and the content of sodium, potassium and calcium estimated using a Zeiss flame photometer type III. Similar estimations were carried out on the reagents and laboratory glassware. The results are given in m-equiv. per kg of fresh tissue.

The material fixed in formalin was cut on a freezing microtome at 30 μ and stained by the Spielmeyer method for myelin and by the cresyl violet method of Nissl. The state of myelination in the first three groups examined was controlled in 5 μ paraffine slices by the osmo-hematoxylin method of SCHULTZE.

RESULTS

In the central nervous system of the foetus (8–9th week of uterine life), in which staining of the nerve fibres by the Spielmeyer and Schultze methods was not yet visible, the water content was high and of a similar level in white and grey cerebral matter and spinal cord. There was also a high content of sodium and calcium in terms of fresh and dry weight. The most characteristic observation for tissues of

TABLE 1.—CONCENTRATION OF WATER, SODIUM, POTASSIUM AND CALCIUM IN THE BRAIN AND SPINAL CORD OF GUINEA PIGS DURING DEVELOPMENT IN M-EQUIV./KG OF FRESH TISSUE

No. of animals in each group: 10. The results are given as mean ± standard error.

	Brain Grey matter				Brain White matter				Spinal cord			
	H_2O%	Na^+	K^+	Ca^{++}	H_2O%	Na^+	K^+	Ca^{++}	H_2O%	Na^+	K^+	Ca^{++}
Foetuses (8–9th week of gestation)	90 ±0·5	156 ± 9	74 ± 7·5	14 ±2·5	91 ±0·5	160 ± 8	61 ± 5	12 ±1	91 ±1	134 ± 8	51 ± 2	12 ±2·5
Animal age												
3 days	89 ±1	87 ±12	69 ±10	6 ±0·7	83 ±1	74 ± 8	77 ±10	6 ±0·2	87 ±1	74 ± 8	43 ± 7	8 ±1
5 days	87 ±1·5	91 ± 9	84 ±12	8 ±0·7	82 ±1	95 ± 8	82 ±12	8 ±0·2	83 ±1	87 ± 4	77 ± 7	11 ±1·5
9 days	86 ±0·5	87 ± 8	90 ± 7	8 ±0·7	80 ±0·5	95 ±13	87 ±15	8 ±0·2	82 ±1	65 ±13	59 ±12	10 ±1
12 days	86 ±0·5	81 ± 4	92 ± 7	6 ±0·7	80 ±2	87 ±17	94 ±12	9 ±1	81 ±1	87 ±13	79 ± 7	11 ±1
2 months	86 ±1	78 ± 4	79 ± 5	8 ±1	74 ±1	117 ±17	79 ±12	9 ±1	79 ±1	65 ±13	64 ±12	12 ±1
8–10 months	82 ±2	70 ±19	69 ±10	7 ±0·5	70 ±1	95 ± 8	87 ±10	9 ±1	74 ±1	78 ± 8	71 ± 7	12 ±1

the foetal central nervous system was that the sodium content was greater than the potassium content. This was statistically significant in white matter and spinal cord, but less pronounced in cerebral grey matter (Table 1). The concentrations of the cations examined were similar in cerebral grey and white matter and in spinal cord.

Slides stained with Spielmeyer hematoxylin of tissues from 3-day old animals

VOL. 5 1959/6

showed marked myelination of the nerve fibres in the spinal cord and of a large number of the cerebral nerve fibres, especially those in the philogenetically older parts. Compared with values obtained for foetuses, the 3-day old animals had a considerably lower content of water in the cerebral white matter, and a lower content of sodium and calcium in the cerebral grey and white matter and spinal cord. These differences were most marked in cerebral white matter. On the other hand, the content of potassium remained at the foetal level. The considerable decrease in the sodium content led to an inversion of the sodium–potassium ratio as compared with the final period of foetal life. When calculated in terms of fresh weight of tissue, the contents of the constituents examined were similar for the different parts of the nervous system except that the content of water was less in white matter than in grey matter and spinal cord (Table 1). When calculated in terms of dry tissue, a significantly lower content of calcium was found in cerebral white matter than in the spinal cord.

The slides of the brain and spinal cord from 5-day old guinea pigs, stained by the Spielmeyer method, showed only negligible differences in myelination as compared with slides from the 3-day old guinea pigs. On the other hand the slides prepared from animals of the age groups 9 days, 12 days and 2 months were not distinguishable from adult brains. The values for the inorganic constituents examined did not change significantly in the period from 3 to 12 days of life, with the exception of an increase in the calcium content in white matter and a decrease in the water content in the spinal cord. The mean sodium–potassium ratio was greater than 1 in all groups, but the difference between sodium and potassium content was significant in some groups only. Chemical determinations in 2-month old guinea pigs showed a further decrease in the water content of cerebral white matter. The content of inorganic ions remained at the same level. The concentrations of the constituents examined in the nervous system of 2-month old and adult animals showed a smaller water content in cerebral white than in grey matter. On the other hand the sodium, potassium and calcium contents were similar in cerebral white and grey matter and in the spinal cord (Table 1).

DISCUSSION

The changes in the water content of the nervous tissue of the guinea pig during development consisted in a decrease, which was most marked in the white matter during the short period between the end of foetal life and the first days of extrauterine life. These results are similar to observations on other species (e.g. DONALDSON and HATAI, 1931). This developmental decrease in the water content, together with the lower content in white than in grey matter of the adult animal indicate that myelin contains less water than do other structural components of nervous tissue. Differences in the amount of water present in tissues should be borne in mind when comparing the concentrations of inorganic ions in animals of different age groups.

The greatest differences in the contents of inorganic ions were found in cerebral and spinal cord tissue between the last period of foetal life and the first few days of extrauterine life. A marked decrease in sodium and calcium content occurred during the period of myelination. In the period of transition from foetal to extrauterine life a decrease in the concentration of sodium, a typical extracellular cation, occurred in white as well as in grey matter of the brain and in the spinal cord. Changes in the sodium content were also significant when expressed in terms of dry weight. This

indicates that changes in the sodium content cannot be exclusively connected with a decrease in the water content. The fall in sodium content should therefore be considered as one of the primary chemical developmental phenomena. Although the fall in sodium and calcium occurs in guinea pigs during the period of myelination, it cannot depend exclusively on this process since it occurs to the same extent in cerebral grey matter where nerve cells predominate over the myelinated nerve fibres.

During extrauterine life no significant changes in the concentrations of the inorganic ions examined took place, in spite of marked changes in the water content. An exception was the increase in calcium concentration in white and grey cerebral matter between the third and fifth day of life. However, the level did not reach that of the last period of foetal life.

The question of the potassium concentration during the early development of the central nervous system should be considered separately. The steady concentration of potassium, a typical intracellular cation, during development was very striking. There were also no significant differences between the potassium content of cerebral white matter, which consists of a small number of cells, and of grey matter, which contains many nerve cells. In addition there were no significant changes in the content of potassium during myelination. These findings may be explained if it is assumed that most of the potassium which is found in large quantities in myelinated nerve fibres, is present in the fibres themselves, and that very little is present in the myelin sheath.

VOL.
5
1959/6

SUMMARY

1. The water content of the white matter of the guinea pig is higher in the foetus than after birth.

2. During early extrauterine development the percentage of water in the brain and in the spinal cord decreases.

3. The sodium and calcium content is higher in the foetal brain and in the spinal cord than in the corresponding tissues taken during extrauterine life.

4. A decrease in sodium and calcium concentration in the tissues of the central nervous system occurs during the period of transition from foetal to extrauterine life which is also the decisive period of myelination.

5. The potassium level in white matter is constant during the period of myelination and during extrauterine life.

REFERENCES

DONALDSON H. and HATAI S. (1931) *J. comp. Neurol.* **53,** 263.

SPERRY W. (1955) In *Neurochemistry* (Edited by ELLIOTT K. A. C., PAGE I. H. and QUASTEL J. H.) p. 234. Thomas, Springfield, Ill.

Journal of Neurochemistry, 1960, Vol. 5, pp. 109–113. Pergamon Press Ltd. Printed in Northern Ireland

INCORPORATION OF RADIOACTIVITY INTO PROTEINS OF THE CEREBRUM, CEREBELLUM AND OTHER ORGANS, AFTER THE INJECTION OF ^{35}S IN THE FORM OF SODIUM SULPHATE*

J. KRAWCZYŃSKI, S. WISZNIOWSKA and I. DREWNOWSKA
Central Clinical Laboratory of the 1st Clinical Hospital in Lublin, Poland

(*Received* 8 *June* 1959)

OL.
5
9/60

THE incorporation of radioactive sulphur, administered as sulphate, into the proteins of the blood serum and of various organs has been investigated by MACHLIN, PEARSON, DENTON and BIRD (1953), DZIEWIATKOWSKI and DI FERRANTE (1957), CHAPEVILLE and FROMAGEOT (1957) and by PEARSON (1956). CHAPEVILLE and FROMAGEOT (1957), and ASTUDILLO and co-workers (1957) in investigations of the chick embryo, showed that the metabolism of inorganic sulphate proceeds through the following stages:

1. Reduction of sulphate to sulphite.
2. Synthesis of cysteic acid.
3. Decarboxylation of cysteic acid to give taurine.

PEARSON (1956) observed that in the chickens inorganic sulphur can be incorporated into cystine, but there is no evidence that the young pig can utilize it for the synthesis of cystine or methionine. According to PEARSON the rabbit has a greater ability to utilize inorganic sulphur for the synthesis of organic molecules than has the pig.

The utilization of inorganic sulphur by nervous tissues has received relatively little attention. RINGERTZ (1956) investigated the uptake of ^{35}S in the brain, after intraperitoneal injection of sodium [^{35}S]sulphate and found that radioactivity could be detected in the brain after 3 hr: after 96 hr the radioactivity in the brain was greater than in the blood serum.

In our investigations we have studied the mechanism of the incorporation of ^{35}S into the proteins of the mouse cerebrum, cerebellum, liver, heart and kidney after the intraperitoneal injection of labelled sodium sulphate. Experiments were also carried out in which labelled sodium sulphate was injected intracranially in the rabbit. In these experiments the uptake of ^{35}S in protein fractions of different parts of the central nervous system was also investigated.

EXPERIMENTAL

Experimental material. The experiments were carried out on white mice weighing 20–25 g in experimental groups containing 10 animals, and on rabbits weighing 1800–2000 g in experimental groups of 3–4 animals.

Methods. The labelled sodium sulphate was administered to mice by intraperitoneal injection (100,000 counts/min per g). The animals were killed at 1, 5, 18, 24, 36, 192 and 264 hr, and protein from the cerebrum, cerebellum, liver, kidney and heart, as well as the serum protein, was isolated according

* This work was supported by the Biochemical Committee of the Polish Academy of Sciences.

to a procedure described previously (KRAWCZYŃSKI, 1958). The radioactivity of the faeces and urine was also measured.

In the second series of experiments labelled sodium sulphate was administered to rabbits by intracranial injection (2,000 counts/min per g). The rabbits were killed after 12, 48 and 120 hr. Whole protein and protein fractions were isolated as described in previous papers (KRAWCZYŃSKI, 1958; KRAWCZYŃSKI and SILICZ, 1957; KRAWCZYŃSKI *et al.*, 1958). Radioactivity was measured in the cerebrum, cerebellum, medulla oblongata, liver, heart, kidney, serum, faeces and urine. The following 4 fractions were separated:

Fraction A: protein dissolving in 0·14 M-NaCl at pH 4·2
Fraction B: protein dissolving in 0·14 M-NaCl at pH 6·7, but not dissolving at pH 4·2
Fraction C: protein dissolving in M-NaCl
Fraction D: protein not dissolving in M-NaCl

The relative specific activities (RSA) of the proteins were calculated as follows:

(i) For the tissue proteins:

$$\text{RSA} = \frac{\text{counts/min per 10 mg protein}}{\text{counts/min per 0·1 ml 'soluble fraction'}}$$

(ii) For the serum protein, faeces and urine:

$$\text{RSA} = \frac{\text{counts/min per 10 mg}}{\text{counts/min per g}} \times 100$$

The 'soluble fraction' was prepared as follows: 1 g tissue was homogenized with 3 ml of 96% (w/v) ethanol according to the procedure for blood serum described by GIRI, KRISHNAMURTHY and VENKITASUBRMANIAN (1952) and left overnight at room temperature. The precipitate then obtained by centrifugation was re-extracted twice more with 60% ethanol. The pooled ethanol extracts were evaporated to dryness on a water bath, and the residue extracted for 24 hr with 10 ml water under conditions preventing evaporation of the fluid. After the removal of the insoluble residue by centrifugation, 0·3 ml of the supernatant was placed on an aluminium disc, evaporated to dryness and its radioactivity measured: 0·1 ml of the 'soluble fraction' was equivalent to 10 mg of tissue.

The effect of the intracranial injection of sodium sulphate on the behaviour of the rabbit. At 2–5 min after the injection of labelled sodium sulphate we observed the onset of convulsions persisting for 1–2 min, after which the behaviour of the rabbits was normal.

RESULTS

The results of typical experiments are shown in Tables 1 and 2 and in Fig. 1a, b, c, d, e and f, which give the relative specific activities (RSA) of the proteins.

TABLE 1.—CHANGES IN THE RELATIVE SPECIFIC ACTIVITY (RSA) OF THE WHOLE PROTEINS OF THE CEREBRUM CEREBELLUM, HEART, LIVER AND KIDNEY OF THE MOUSE AFTER THE INTRAPERITONEAL INJECTION OF SODIUM [^{35}S]SULPHATE.

Tissue	Time after injection (hr)						
	1	5	18	24	36	192	264
Cerebrum	13·85	38·87	43·95	54·19	54·89	18·63	13·18
Cerebellum	4·14	14·88	87·50	21·55	7·11	6·94	5·00
Spinal cord	4·42	12·97	31·06	12·20	7·31	3·42	2·26
Heart	12·18	23·06	23·79	23·54	20·73	12·93	5·84
Liver	26·72	86·64	24·62	27·28	28·91	19·69	5·59
Kidney	10·25	29·38	20·23	10·36	9·32	5·72	1·85
Blood serum	5·32	3·78	0·46	0·56	0·23	0·05	0·03

VOL. 5 1959/6

TABLE 2.—THE RELATIVE SPECIFIC ACTIVITY IN THE FAECES AND URINE OF THE MOUSE AFTER THE INTRAPERITONEAL INJECTION OF SODIUM [^{35}]SULPHATE.

	Time after injection (hr)						
	1	5	18	24	36	192	264
Faeces	11·08	26·15	40·85	6·00	0·44	0·10	0·10
Urine	121·00	542·20	98·00	33·00	1·01	0·70	0·05

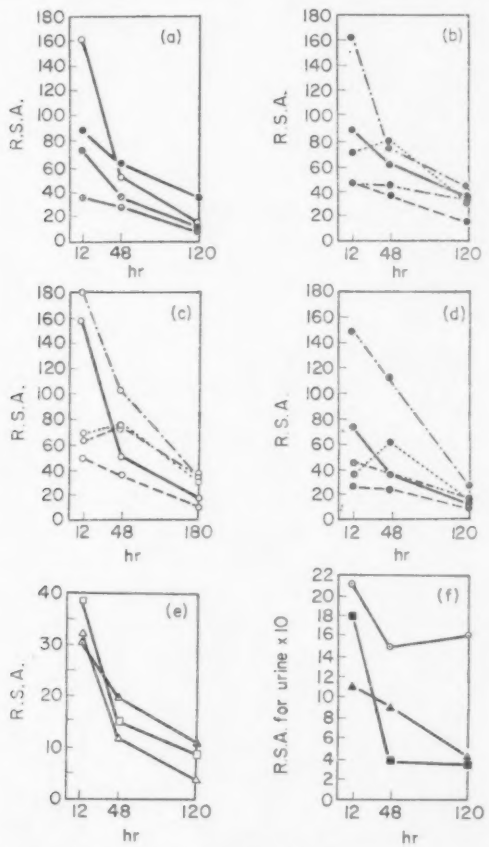


FIG. 1.—Changes in the relative specific activity (RSA) of tissue proteins and protein fractions of faeces and urine of the rabbit after intracranial injection of sodium [^{35}S]sulphate.
(a) Whole protein of the cerebrum, cerebellum and medulla oblongata.
(b) Brain: grey matter (fractions)
(c) Brain: white matter (fractions)
(d) Cerebellum (fractions)
(e) Whole protein of the liver, heart and kidney.
(f) Faeces, urine and blood serum.
———Whole tissue proteins, – – – – – fraction A, — · — · — · fraction B, ... fraction C, — — · · — — · · — — · · fraction D.
Rabbits: weight 1800–2000 g Sodium [^{35}S] sulphate: 2000 counts/min/g
● — grey matter, ○ — white matter, ⊗ — cerebellum, ⊕ — medulla oblongata, △ — heart, □ — liver, △ — kidney, ■ — blood serum, ▲ — faeces, ○ — urine.

From the results of these experiments the following conclusions can be drawn:

(a) After intraperitoneal injection of labelled sulphate, the radioactivity very soon penetrates the 'blood-brain barrier' and enters the proteins of the brain. The RSA of the brain protein is relatively high, lower only than the RSA of the liver protein (Table 1). After 18 hr, the highest RSA is found in the protein of the cerebellum.

(b) After the intracranial injection of sodium [^{35}S]sulphate, the highest RSA is found in the protein of the cerebral white and grey matter. Lower RSAs are given by the protein from the cerebellum and medulla oblongata. Under these experimental conditions the RSAs of the proteins from the liver, heart and kidney are the lowest (Fig. 1). Of the different protein fractions, the highest RSA is found in fraction B, and, in decreasing order of activity, in fractions C, D and A.

(c) The proteins of the blood serum show a remarkable decrease in RSA during the first 5 hr after the intraperitoneal injection of labelled sulphate. After intracranial injection a decrease in the RSA occurs between 12 and 48 hr. After 48 hr the RSA is relatively low and there is no further decrease.

(d) After intracranial as well as after intraperitoneal injection, most of the radioactivity is found in the urine, especially during the first 5 hr. After 12 hr the radioactivity of the urine decreases slowly.

(e) The radioactivity of the faeces is always low, and it decreases slowly.

VOL. 5 1959/6

DISCUSSION

The main problem under discussion is the extent of the utilization of inorganic sulphur in higher animals for the synthesis of organic compounds and especially of tissue proteins. It is known that inorganic sulphur may be used for the synthesis of sulphur-containing amino acids and amines (MACHLIN *et al*, 1953; PEARSON, 1956; CHAPEVILLE and FROMAGEOT, 1957) and in this way indirectly for the synthesis of proteins as well as for the synthesis of chondroitin sulphuric acid and related substances. It appeared possible that the inorganic sulphur might be physically attached to the protein molecules and not chemically combined: but this possibility can be excluded, for our experimental conditions since control experiments were carried out in which brain homogenates were incubated with labelled sodium sulphate for 1, 5 and 12 hr and then treated in exactly the same way as the tissue obtained from the experimental animals that received radioactive sulphur by intracranial or intraperitoneal injection. In such experiments almost the whole of the radioactivity was found in the 'soluble fraction' (more than 98 per cent) and the radioactivity of the protein was negligible.

The very high radioactivity in the urine, especially during the first 5 hr, indicates that most of the sulphate is excreted unchanged. This is in agreement with the earlier observations of EVERETT and SIMMONS (1952) and WALSER, REID and SOLDIN (1953), who found that more than 80 per cent of injected sulphate is excreted in the urine during the first 24 hr.

The remainder of the injected sodium sulphate may be used for the synthesis of sulphur-containing amino acids and chondroitin sulphuric acid, and in these forms incorporated into the proteins. This view is partially confirmed by the fact that the nucleoprotein-containing fractions B and C, which probably play a part in the synthesis of tissue proteins, show the highest RSA, as after administration of [^{35}S]methionine or [^{14}C]tyrosine (KRAWCZYŃSKI and SILICZ, 1957; KRAWCZYŃSKI, 1958).

The differences in the radioactivity of the different protein fractions after injection of [^{35}S]methionine and of [^{35}S]sulphate may be due to the fact that [^{35}S]sulphate is incorporated also in chondroitin sulphuric acid and related compounds, which are present in different proportions in the individual protein fractions.

SUMMARY

Sodium [^{35}S]sulphate was injected intraperitoneally in white mice (100,000 counts/min per g) and the animals were killed after 1–264 hr. The radioactivity of proteins isolated from the cerebrum, cerebellum, liver, kidney, heart and serum, as well as the radioactivity of the faeces and urine, were measured. In experiments with rabbits [^{35}S]sulphate was injected intracranially: in these experiments proteins of the grey and white matter of the brain and of the cerebellum were fractionated with 0·14 M-NaCl and 1M-NaCl.

During the first 5 hr after intraperitoneal injection of [^{35}S]sulphate, the highest specific radioactivity was found in the proteins of the liver and, in decreasing order, in proteins of the cerebrum, heart and kidneys; it was lowest in the proteins of the cerebellum and spinal cord. After 18 hr, the highest RSA was found in the proteins of the liver, heart and kidney.

After intracranial injection of sodium [^{35}S]sulphate, the highest RSA was in the protein of the cerebral white and grey matter and cerebellum, and the lowest in the proteins of the livers, heart and kidney. Of the different protein fractions, the highest RSA was found in the nucleoprotein-containing fractions B and C.

Most of the radioactivity was eliminated in the urine within the first few hours.

The problem of the utilization of inorganic sulphate for the synthesis of organic compounds in higher animals was discussed.

Acknowledgement—We are very grateful to Prof. D. RICHTER for his help in preparation of the manuscript.

REFERENCES

ASTUDILLO M., SANZ F., SANZ B. and VALDECANTOS A. (1957) *Bull. Soc. Chim. biol., Paris* **39,** 569.
CHAPEVILLE F. and FROMAGEOT P. (1957) *Biochim. biophys. acta* **26,** 538.
DEYRUP I. and USING H. (1955) *J. gen. Physiol.* **38,** 599.
DZIEWIATKOWSKI D. and DI FERRANTE N. (1957) *J. biol. Chem.* **227,** 327.
EVERETT N. and SIMMONS B. (1952) *Arch. Biochem. Biophys.* **35,** 152.
GIRI K., KRISHNAMURTHY K. and VENKITASUBRMANIAN T. (1952) *Lancet* **263,** 562.
KRAWCZYŃSKI J. (1958) Paper presented at the 3rd Neurochemical Symposium.
KRAWCZYŃSKI J., DREWNOWSKA J., FLESZYNSKI J. and RYCAJ M. (1958) *Acta Biochim. Polon.* **5,** 139.
KRAWCZYŃSKI J. and SILICZ T. (1957) *Ukr. biochem. J.* **29,** 25.
MACHLIN L., PEARSON P., DENTON C. and BIRD H. (1953) *J. biol. Chem.* **205,** 213.
PEARSON P. (1956) In *La Biochimie du Soufre*, p. 153. Colloques Internationaux du Centre Nationale de la Recherche Scientifique, Paris.
RINGERTZ N. (1956) *Expt. Cell. Res.*, **10,** 230.
SALMON W. and DAUGHADAY W. (1958). *J. Lab. clin. Med.* **51,** 167.
WALSER M., REID A. and SOLDIN D. (1953). *Arch. Biochem. Biophys.* **45,** 91.

Journal of Neurochemistry, 1960. Vol. 5, pp. 114–120. Pergamon Press Ltd. Printed in Northern Ireland

A HISTOCHEMICAL CONTRIBUTION TO THE STUDY OF THE MOLECULAR MORPHOLOGY OF MYELIN SHEATH*

MOSHE WOLMAN† and SARAH HESTRIN-LERNER
Departments of Pathology and Physiology, The Hebrew University, Hadassah Medical School, Jerusalem, Israel

(*Received* 10 *June* 1959)

VOL. 5 1959/6

THE myelin sheath of nerves is a highly organized, almost crystalline, structure. Data regarding the spatial relations of the various constituents of myelin have been obtained by the use of polarized light (SCHMIDT, 1936), by electron microscopy (FERNANDEZ-MORAN, 1957), by X-ray diffraction (SCHMITT *et al.*, 1941; ELKES and FINEAN, 1953; FINEAN, 1953; FINEAN and ROBERTSON, 1958) and by chemical methods in which two or more components of the sheath were extracted together (KONNIKOVA, 1950; FOLCH and LEES, 1951; FOLCH *et al.*, 1951; LEBARON and FOLCH, 1956; see also LEBARON and FOLCH, 1957). On the strength of the results, models for the molecular morphology of the sheath have been proposed by some investigators, the most recent being those of KLENK (1953) and of FINEAN (1957).

The purpose of this paper is to report the results of a histochemical investigation of the normal myelin sheath of rat sciatic nerve and to suggest some modifications to the proposed models of the molecular morphology of myelin.

MATERIALS AND METHODS

Adult albino rats under ether anaesthesia were exsanguinated by cutting the aorta and vena cava inferior. The sciatic nerves were dissected out, frozen in a cryostat (Coons model) at from $-15°$ to $-20°$ and cut longitudinally at 8 μ thickness. Sections were mounted on albuminized slides, were partly dried in the cryostat for 30–60 min and were then allowed to thaw and dry at room temperature for a few minutes. The unfixed sections were either treated immediately, or after the slides had been kept for some hours in a refrigerator. Stained sections, as well as unstained sections (to be examined also with ordinary and with polarized light), were prepared from the frozen and dried material. Prior to staining or direct observation, some of the sections were subjected to the following procedures: (1) extraction with lipid solvents (ether, acetone, and methanol–chloroform, (1 : 2)) at different temperatures; (2) digestion with trypsin for 3 hr at 37°; (twice crystallized trypsin, Nutritional Biochemicals Corp. 1 mg/ml—1 ml; 0·2 M-phosphate buffer, pH 7·8—8 ml; NaF 1% w/v—1 ml); (3) heating in an oven at 100° for one hr; (4) combinations of the above mentioned treatments.

The following staining methods were used: (1) hematoxylin and eosin (HE); (2) periodic acid-Schiff (PAS); (3) the Bi–Col method (WOLMAN, 1956); (4) Sudan black (B); (5) performic acid-Schiff (PFAS); (6) Schultz's method for cholesterol.

RESULTS

The myelin sheath in the native state

In sections stained with HE the myelin sheath appeared markedly basophilic or amphophilic. The sheath was bi-refringent, it stained pale blue with the Bi–Col

* This study was supported by Grant No. 138R of the National Multiple Sclerosis Society, New York; a short account of this communication was presented to the 4th International Congress of Biochemistry in Vienna, in September, 1958.

† Present address: Department of Pathology, Government Hospital, Tel-Hashomer, Israel.

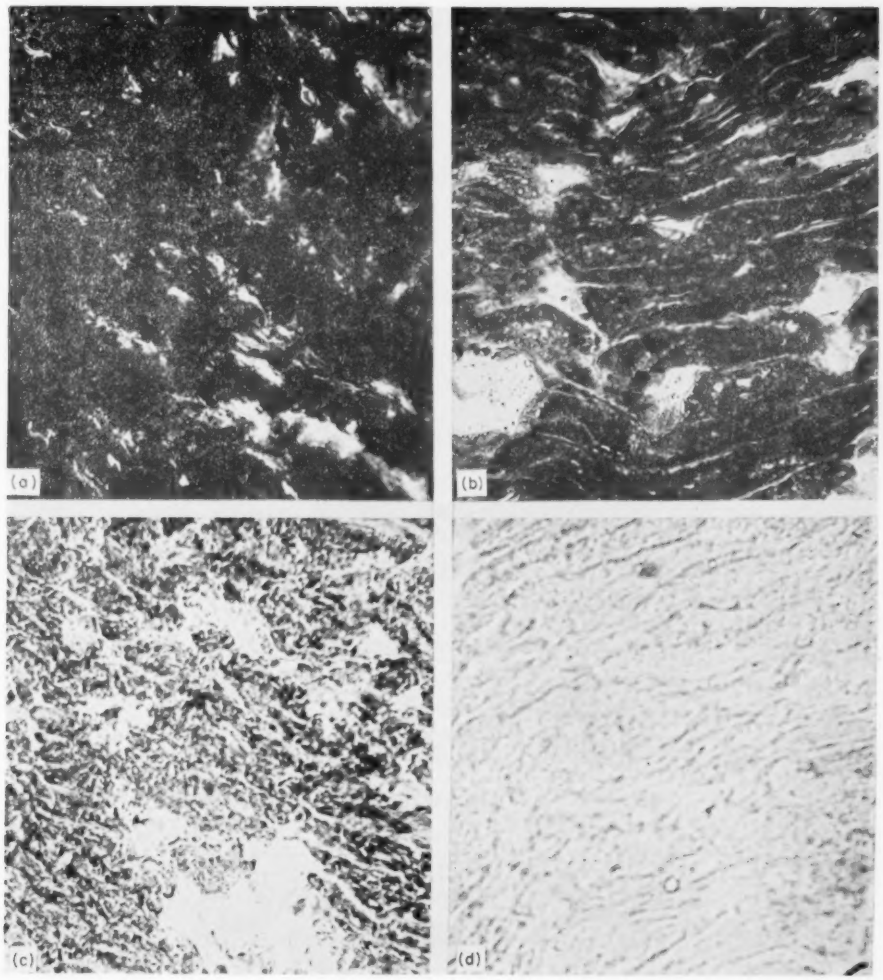


FIG. 1.—Fresh frozen sections of rat sciatic nerve stained with Sudan black B. All the sections were stained simultaneously and photographed and printed under identical conditions. ×565.

(a) Untreated section. (b) Section treated with acetone for one hr at +4°. (c) Section treated for one hr with ether at −20°. *D* Section treated for one hr with methanol–chloroform (1 : 2) at +4°.

Note the reduction of staining intensity from (a) to (d) and the collapsed and linear appearance of myelin in (d).

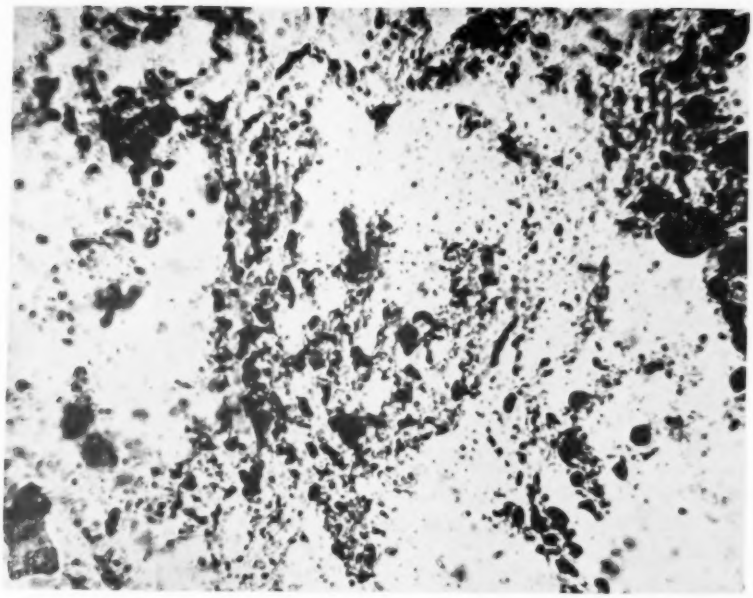


FIG. 2.—Fresh frozen section of rat sciatic nerve digested with trypsin and then stained with Sudan black B. ×565.
Note the lightly stained sheaths and the heavily stained droplets and myelin figures.

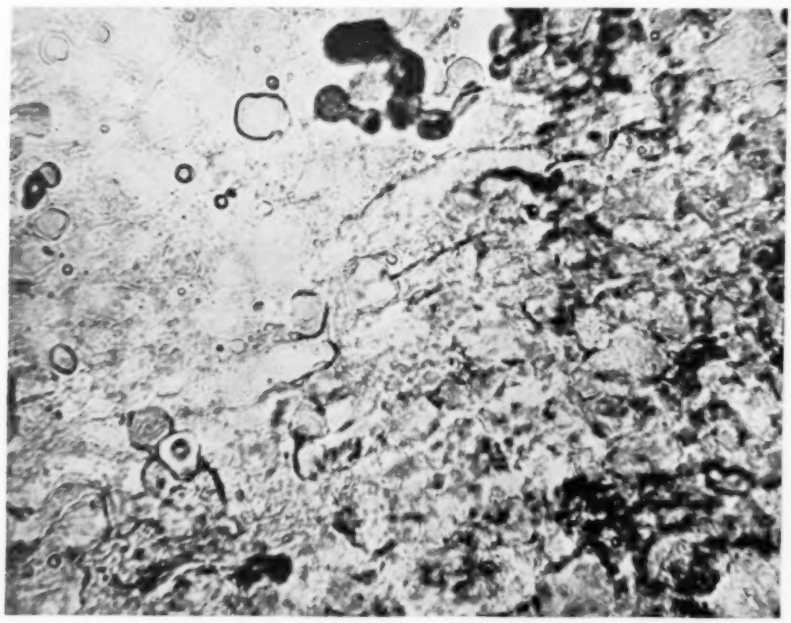


FIG. 3.—Fresh frozen section of rat sciatic nerve digested with trypsin and then stained with PAS. ×520.
Note the lightly stained sheaths and the heavily stained droplets and myelin figures.

VOL.
5
1959/6

method, and was intensely stained with the PAS and PFAS procedures. It stained bluish-grey with Sudan black (Fig. 1a), and sections which were stained with Sudan black showed the dichroic effect (bronze colour), which has been described and studied by DIEZEL (1957), when observed under polarized light. There was a strong and regular staining of the sheaths with SCHULTZ's method for cholesterol.

Effects of lipid solvents

Acetone at 4°. Prior treatment of sections (mounted on slides) for one hr at about +4° with acetone had no effect on the basophilia, the birefringence, and the blue staining with Bi–Col. With PAS, PFAS and Sudan black the intensity of staining was somewhat decreased; however, the dichroic effect persisted; the Schultz reaction became completely negative.

Ether at from −15° *to* −20°. Treatment of sections with ether at from −15° to −20° for one hr, had similar, but not identical effects. The basophilia, the blue staining with Bi–Col and the birefringence were not markedly affected and, as with acetone, the Schultz reaction became negative but the reduction in staining with Sudan black, PAS and PFAS was more marked.

Ether at +30°. Treatment with ether at 30° for one hr had more pronounced effects. There was marked reduction in birefringence, basophilia, as well as in the staining with Sudan black, PAS and PFAS. The dichroic effect with polarized light in the sections treated with Sudan black was markedly reduced or abolished. With the Bi–Col procedure, the myelin in most places appeared unstained. In other areas it appeared a very pale blue. With all the staining procedures the sheaths appeared to have collapsed in some areas and were represented by narrow lines lying attached to the neurilemma.

Methanol–chloroform at 4°. Treatment with methanol–chloroform (1 : 2) at 4° for one hr had marked effects on the morphology and staining characteristics. In HE stained sections the sheath became acidophilic or faintly amphophilic, and was mostly represented by a narrow collapsed line at the periphery of the area which had previously been occupied by the myelin. There was no birefringence, and no staining with Schultz's method. In most places the myelin remained unstained with the Bi–Col method, but occasionally there were areas which stained pale blue or pale brown red. The staining with Sudan black, PAS and PFAS was very pale and mostly in the form of a narrow line. Fig. 1 shows the effects of lipid solvents on staining with Sudan black.

EFFECTS OF TRYPSIN AND TRYPSIN FOLLOWED BY SOLVENTS

Trypsin only

Sections incubated for 3 hr with trypsin, and then rinsed with distilled water and stained, differed markedly from non-trypsinized sections. In HE stained sections the sheath appeared rather narrow and palely basophilic. It stained pale blue with Bi–Col. With Sudan black, the sheath itself appeared as a lightly stained band but in addition a great number of lipid droplets and myelin figures were found within the nerve fibres and around them. These were of various sizes, sudanophilic, often anisotropic and dichroic (in the larger droplets only the peripheral part was markedly sudanophilic). Sections stained with PAS and PFAS also showed reduced staining of the sheaths

and the presence of droplets which stained more intensely at the periphery. Schultz-stainable material was distributed unevenly, mostly in the droplets, but occasionally within the sheaths. Figs. 2 and 3 show the appearance of trypsinized sections.

Trypsin followed by lipid solvents

Treatment with cold ether, acetone or methanol–chloroform of previously trypsinized sections had the following effects: staining of the sheath itself with Sudan black, PAS and PFAS was further reduced; the lipid droplets disappeared completely, and the Schultz reaction became negative. The basophilia and the blue-staining with Bi–Col were almost completely abolished (the sheaths became palely eosinophilic). Trypsinized sections which were subsequently extracted with methanol–chloroform differed markedly from sections in which tryptic digestion was followed by extraction with ether or acetone. Whereas after ether and acetone extractions the sheaths were either unstained or had a barely perceptible bluish tint with Bi–Col, methanol–chloroform extraction of trypsinized sections caused the appearance of intense red-brown staining. Ether or acetone treatment of trypsinized sections did not markedly reduce the staining with PAS and PFAS of the sheaths, but extraction with methanol–chloroform of such sections caused almost complete disappearance of these reactions.

VOL.
5
1959/6

Effects of heat

The appearance of dried frozen sections which were kept in an oven at 100° for one hr was in some respects similar to that of trypsinized sections. The sheaths themselves were sudanophilic, baso- or amphophilic, stained blue with Bi–Col, positive with PAS and PFAS, but they were studded with droplets in which only the periphery stained with Sudan black, PAS and PFAS whereas the interior was positive only with Schultz's method and was birefringent.

Treatment with lipid solvents of the heat-treated sections produced effects similar to those which were obtained by extracting trypsinized sections (Fig. 4). Comparable results were also obtained by treating dried sections directly for one hr in hot solvents (methanol–chloroform or acetone at 60°). In these experiments the sections had similar staining characteristics to those obtained by exposing the sections for one hr at 100°, followed by treatment with the same solvents at low temperature. However, unstained sections processed in the two ways were not similar; those exposed to hot solvents appeared opaque in transmitted light because of an irregular precipitation, probably of crystalline nature (Figs. 5 and 6).

DISCUSSION

The histochemical methods

The histochemical methods used in this study indicate the presence of the following compounds. Basophilia shows the presence of negatively charged (acidic) compounds (SINGER, 1952). The Bi–Col staining method differentiates weakly acidic compounds, which stain red to brown, from strongly acidic ones, which stain blue (WOLMAN, 1956). The blue staining with Bi–Col is thus comparable to metachromatic staining at low pH levels (2 or below), and the red staining to metachromasia at pH 5–6. The PAS method stains 1 : 2-glycols or 1-hydroxy 2-amino groups, and therefore mainly carbohydrate moieties (LILLIE, 1954) and to a lesser extent reactive ethylenic links

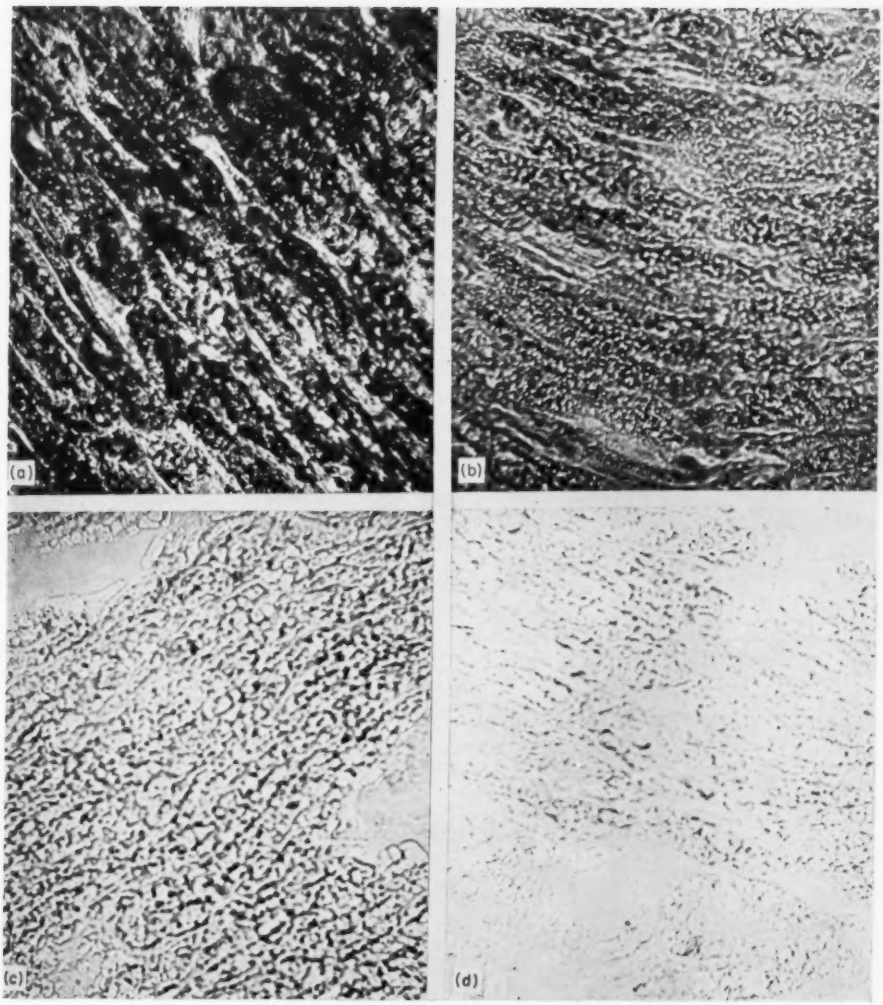


FIG. 4.—Fresh frozen sections of rat sciatic nerve stained with Sudan black B. All sections were kept in an oven at 100° for one hr. Conditions of staining, photography and printing were identical for all sections. ×565.

(a) Heated and stained section. (b) Section treated with acetone at +4° for one hr after heating. (c) Section treated with ether at −20° after heating. (d) Section treated with methanol–chloroform at +4° after heating.

Note the formation of darkly stained droplets and masses in (a), most of which remained within the sheaths. The contents of these droplets were dissolved by all lipid solvents. The intensity of staining of the sheaths decreases from (b) to (d).

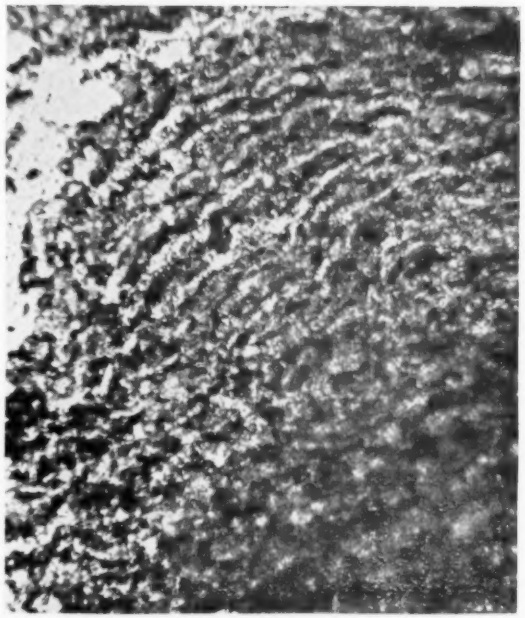


Fig. 5—×400.

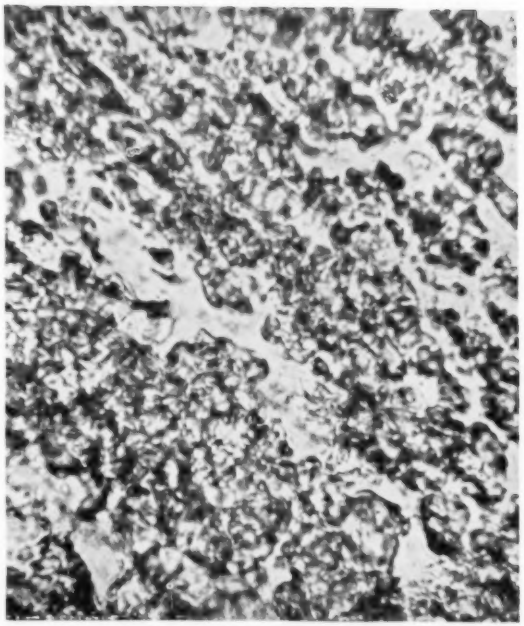


Fig. 6—×1600.

FIGS. 5 and 6.—Fresh frozen section of rat sciatic nerve treated for one hr in ethanol at 60°. Unstained. Note the precipitation of an opaque, probably crystalline, material.

VOL.
5
1959/6

(WOLMAN, 1950). These ethylenic links, which are present mainly, or only, in unsaturated lipids, are stained by the PFAS procedure (LILLIE, 1954). The Schultz procedure is considered to be specific for unsaturated sterols and would therefore indicate the presence of cholesterol. Sudan black stains lipids because of its high partition coefficient between non-polar and polar media.

The phospholipids

In the experiments described above, the basophilia and the blue staining with Bi–Col were due mainly to phospholipids. This is concluded from the effects of solvents and is also indirectly confirmed by some data in the literature. The studies of WISLOCKI and SINGER (1950) have shown that the metachromasia of myelin in frozen sections is due to a lipid which is extracted in the preparation of paraffin sections; the work of LANDSMEER (1951, 1955) shows that the metachromasia of myelin is due to lipidic phosphate groups. It has also been repeatedly pointed out that the staining with colloidal iron, which results in the blue staining in the Bi–Col procedure, is comparable to metachromasia (MANCINI, 1950; GOMORI, 1952; WOLMAN, 1958) and it may therefore be assumed that phospholipids are responsible for both. DIEZEL (1957) has shown that the dichroic effect of Sudan black is also due to phospholipids. This is borne out by our experiments.

Effect of lipid solvents on the structure of the sheath

The finding that lipid solvents and especially cold methanol–chloroform caused a collapse of the myelin to a narrow band indicates that parts of the structure which maintain the radial arrangement of myelin have been extracted, so that the concentric layers collapse. It is remarkable that similar findings were reported by electron microscopists (FERNANDEZ-MORAN and FINEAN, 1957).

Effects of tryptic digestion

Treatment by trypsin caused displacement of a considerable part of the myelin lipids which coalesced to form droplets. The droplets were free and could be completely extracted by any of the lipid solvents tested. Those lipids which were not displaced by treatment with trypsin were PAS and PFAS positive and could be effectively extracted by methanol–chloroform only. It is therefore probable that the lipid remaining in place after tryptic digestion consists of cerebrosides, either alone or together with other lipids which are not easily dissolved by acetone or ether.

Unmasking of the weakly acidic groups

In this study, red staining with the Bi–Col procedure appeared in unfixed frozen sections only after tryptic digestion or heating followed by methanol–chloroform extraction. However, in a previous study (WOLMAN, 1957) red-brown staining of myelin with Bi–Col was observed in ethanol-fixed paraffin sections which were not trypsinized. Since the preparation of paraffin sections entails exposure both to heat and to lipid solvents, both the findings are readily reconciled.

The weakly acidic groups unmasked by tryptic digestion followed by methanol–chloroform extraction seem to belong to the myelin polysaccharide (myelosaccharide) since the only extractable fraction of nervous tissue which stains red with Bi–Col is a hexosamine-containing polysaccharide (WOLMAN, unpublished).

The fact that the weakly acidic groups (probably carboxylic) of the polysaccharide can be detected only after tryptic digestion followed by methanol–chloroform extraction indicates that the polysaccharide is bound through these groups to a lipid moiety. The solubility and staining characteristics of this lipid suggest it to be a cerebroside or a cerebroside-sphingomyelin structure. These data, and the findings of BRANTE (1957) and of WOLMAN (1959) that a great part of the hexosamine of myelin can be extracted with water after tryptic digestion, show that a complex of protein-lipid-polysaccharide which is trypsin-digestible forms part of the myelin macromolecule. Tryptic digestion, and to a lesser extent denaturation by heat, free the lipo-polysaccharide from the bond connecting it to the protein.

CORRELATION OF THE HISTO-CHEMICAL DATA WITH FINDINGS OBTAINED BY ELECTRON MICROSCOPY AND X-RAY DIFFRACTION

The findings of the present study have a bearing on some aspects of the molecular morphology of the myelin sheath, and accordingly refer to suggested models of the structure of myelin. The model proposed by FINEAN (1957) for the molecular structure

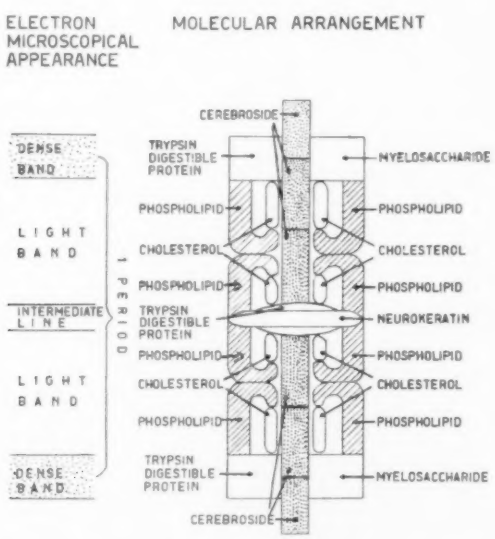


FIG. 7.—Schematic representation of the molecular morphology of myelin. On the left the corresponding appearance in electron microscopy.

of myelin, which is based on data obtained by X-ray diffraction and by electron microscopy, may only be considered as tentative. One of the shortcomings of FINEAN's scheme is that it is two-dimensional, and its transference into a three-dimensional structure would upset the quantitative relations between the constituent moieties. However, with the information at present available, the construction of a three-dimensional model does not appear feasible. The basic lines of FINEAN's model have therefore been retained and the scheme altered in some particulars in accordance with the findings of this study (Fig. 7).

The dense band

In this scheme the dense band of electron microscopists contains the polysaccharide (myelosaccharide) attached through cerebroside to a trypsin-digestible protein. This tallies with a number of observations: (a) the findings of this study which indicate that cerebroside forms at least part of the link between the polysaccharide and the protein; (b) the findings of the extraction experiments which suggest that a hexosamine-containing polysaccharide is bound by a lipid to a trypsin-digestible protein (WOLMAN, 1959); (c) the observation made by electron microscopists that in trypsinized sections the dense band is broken up into rodlike units (FERNANDEZ-MORAN and FINEAN, 1957). These rodlike units would then represent the lipo-polysaccharide moieties remaining after the protein has been digested.

The light band

The collapse of the structure after treatment with lipid solvents, particularly methanol–chloroform, has been observed both here and by electron microscopy. The thin collapsed line, which stained faintly with PAS and Sudan black probably represents the sum of the moieties of the dense band, including cerebroside. Electron micrographs show that the dense bands coalesce after such treatment, with an almost complete disappearance of the interval between them. This means that both the constituents of the intermediate line and the lipids of the light band are extracted by the solvent. Since it is known that cold methanol–chloroform extracts the neurokeratin-containing proteolipid from white matter, it is suggested that neurokeratin might be situated in the intermediate line. The lipid moieties hanging around this protein core give the complex its specific solubility characteristics.

The proposed scheme is a very rough approximation which serves only to bring together data obtained by different techniques. It main points are:

(1) that the dense band represents a protein-lipid-polysaccharide complex which binds osmium in electron micrographs;
(2) that within this band, the weakly acidic groups of the polysaccharide are bound through the lipid (cerebroside or cerebroside + sphingomyelin) to the protein;
(3) that most of the lipid components of myelin are situated in the light bands which absorb little osmium;
(4) that neurokeratin, surrounded by lipid moieties, might be situated in the intermediate line and that the extraction of the whole proteolipid unit causes collapse of the structure.

It is not clear whether the protein-lipid-polysaccharide unit represented in Fig. 7 is a repeating unit. It is believed that the true structure is more complicated, as work which is now in progress indicates that within the macromolecule of myelin other arrangements of the polysaccharide and cerebroside also occur.

SUMMARY

Sciatic nerves of normal rats were cut in the cryostate and subjected to a number of histochemical tests directly and after extractive and digestive procedures. The findings, in correlation with electron-microscopical and X-ray diffraction studies, yield data which are relevant to the spatial relations of the constituents of the myelin sheath.

A modification to the scheme of FINEAN for the structure of myelin has been proposed. It is suggested that the dark band observed by electron microscopy consists of a polysaccharide-lipid-protein complex, in which the lipid is, at least in part, cerebroside, and the protein is trypsin-digestible. The light band contains the bulk of myelin lipids arranged radially; the intermediate line might consist of neurokeratin and some trypsin-digestible protein.

The weak points of the proposed modification as well as of the previous schemes representing the structure of myelin are pointed out.

REFERENCES

BRANTE G. (1957) *Metabolism of the Nervous System* (Edited by RICHTER D.) Pergamon Press, London.

DIEZEL P. B. (1957) *Die Stoffwechselstörungen der Sphingolipoide.* Springer, Berlin.

ELKES J. and FINEAN J. B. (1953) *Exp. Cell Res.* **4,** 82.

FERNANDEZ-MORAN H. and FINEAN J. B. (1957) *J. biophys. biochem. Cytol.* **3,** 725.

FERNANDEZ-MORAN H. (1957) *Metabolism of the Nervous System* (Edited by RICHTER D.) Pergamon Press, London.

FINEAN J. B. (1953) *Experientia* **9,** 17.

FINEAN J. B. (1957) *Metabolism of the Nervous System* (Edited by RICHTER D.) Pergamon Press, London.

FINEAN J. B. and ROBERTSON J. D. (1958) *Brit. med. Bull.* **14,** 267.

FOLCH J. and LEES M. (1951) *J. biol. Chem.* **191,** 807.

FOLCH J., ASCOLI I., LEES M., MEATH J. A. and LEBARON F. N. (1951) *J. biol. Chem.* **191,** 833.

GOMORI G. (1952) *Microscopic Histochemistry, Principles and Practice.* Chicago University Press, Chicago.

KLENK E. (1953) *Naturwissenschaften* **10,** 449.

KONNIKOVA G. S. (1950) *Ukr. biochem. J.* **22,** 3. Abstracted in *Chem. Abstr.* (1954) **48,** 128280.

LANDSMEER J. M. F. (1951) *Acta physiol. néerl.* **2,** 112.

LANDSMEER J. M. F. (1955) *C.R. Ass. Anat.* **42,** 856.

LEBARON F. N. and FOLCH J. (1956) *J. Neurochem.* **1,** 101.

LEBARON F. N. and FOLCH J. (1957) *Physiol. Rev.* **17,** 539.

LILLIE R. D. (1954) *Histopathologic Technique and Practical Histochemistry.* Blakiston, Philadelphia.

MANCINI R. E. (1950) *Rev. Soc. argent. Biol.* **26,** 139.

SCHMIDT W. J. (1936) *Z. Zellforsch.* **23,** 657.

SCHMITT F. O., BEAR R. S. and PALMER K. J. (1941) *J. cell. comp. Physiol.* **18,** 31.

SINGER M. (1952) *Int. Rev. Cytol.* **1,** 211.

WISLOCKI G. B. and SINGER M. (1950) *J. comp. Neurol.* **92,** 71.

WOLMAN M. (1950) *Proc. Soc. exp. Biol., N.Y.* **75,** 583.

WOLMAN M. (1956) *Bull. Res. Council Israel,* **6E,** 27.

WOLMAN M. (1957) *J. Neurochem.* **1,** 370.

WOLMAN M. (1958) *Acta histochem.* Suppl. **1,** 130.

WOLMAN M. (1959) *Proceedings of the Third International Neurochemical Symposium.* To be published.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 121–127. Pergamon Press Ltd. Printed in Northern Ireland

BIOCHEMICAL STUDIES ON PSYCHOTROPIC DRUGS—I

THE EFFECT OF PSYCHOTROPIC DRUGS ON γ-AMINOBUTYRIC ACID AND GLUTAMIC ACID IN BRAIN TISSUE

M. J. E. ERNSTING,* W. F. KAFOE,* W. TH. NAUTA,†
H. K. OOSTERHUIS‡ and C. DE WAART‡
Vrije Universiteit, Amsterdam and Research Department
Brocades, Amsterdam

(*Received* 3 *September* 1959)

OL.
5
9/60

SOME time ago, one of us (DE WAART, 1956, 1958) published the results of an investigation of the inhibitory effect of a number of diphenhydramine§ derivatives on intermediary metabolism. Like the barbiturates, diphenhydramine reduces the oxygen consumption during the aerobic metabolism of rat brain slices (glucose as substrate). The compounds studied at the time mainly consisted of alkyl derivatives of diphenhydramine. Generally speaking, these derivatives display a more pronounced inhibitory effect than diphenhydramine itself. It was also found that some compounds examined affected the amino acid metabolism. By paper chromatography it was demonstrated that at the end of the experiments the concentration in the incubation fluid of some of the amino acids which are released initially (MCILWAIN, 1959) remained at a high level, whereas in the blanks these acids were regained by the tissue. After ninhydrin coloration the chomatograms showed four bands, two of which were particularly distinct. These were identified at the time as glutamic acid and methionine. Barbiturates did not have this effect on the amino acid pattern of the incubation fluid.

Of the compounds investigated, orphenadrine (β-dimethylaminoethyl-2-methylbenzhydrylether, Disipal ®) produced the strongest effect. The pharmacological properties of orphenadrine are very different from those of diphenhydramine. (It has a strongly reduced antihistamine action and a strongly increased atropine one.) The preparation is used for treating Parkinsonism, and recent investigations (SCHEURLE, 1957; ROBITSCHER, 1958; PFEIFFER, 1959) show that it also has psychotropic properties. These observations prompted us to study the effect of psychotropic drugs on amino acid metabolism also. A preliminary note on the results of this investigation was published by us some time ago (NAUTA *et al.*, 1958).

METHODS

Tissue respiration. To measure the respiratory activity of brain slices, Warburg techniques were employed. Adult albino rats weighing about 200 g were decapitated and after the removal of the thalamus, the brain was cut into slices; 80–100 mg in phosphate-saline were taken per Warburg flask.

The medium used contained: 120 mM-NaCl; 1 mM-$MgSO_4$; 19·5 mM-Na_2HPO_4 and 8·5 mM-KH_2PO_4 (pH 7·2). The potassium concentration was somewhat higher than the usual 5 mM, as it had been found that this improved the reproducibility of the results. The main vessel of the Warburg

* Research Department Brocades, Amsterdam.
† Chemical Laboratory Vrije Universiteit, de Lairessestraat 174, Amsterdam.
‡ Laboratory for Chemical Physiology Vrije Universiteit, Valeriusplein 11, Amsterdam.
§ β-dimethylaminoethyl benzhydrylether hydrochloride, a well-known antihistamine.

flask contained 2 ml of medium, and the side arm, 0·5 ml. To the latter, glucose (final concentration 0·02 M) and, if necessary, the drug under test (final concentration 0·002 M) were added. The solution from the side arm was tipped into the main vessel after 10 min equilibration, and 3 min before the first manometric reading was taken. The equilibration for 3 min was followed by 60 min incubation at 37°. The oxygen consumption in μl/mg moist brain tissue was calculated from the difference between the first and the last manometric readings. Any inhibition of the oxygen consumption by a compound was expressed in per cent with respect to the mean blank value obtained in the same experiment. In each experiment, ten Warburg vessels were used, of which two were thermo-barometers, four were blanks and four contained the substance under test. Each experiment was repeated several times with brain slices from different rats.

Determination of the amino acids in the incubation fluid. After the incubation, the contents of the Warburg vessels were centrifuged. The supernatant fluid was fractionated by ascending paper chromatography with *n*-butanol–acetic acid–water (60 : 15 : 25 v/v) as the solvent. The dimensions of the paper strips (Schleicher und Schüll 2043 B) were 7 × 20 cm. Desalting the supernatant fluid proved to be unnecessary, and 0·06 ml was applied directly to the strips, 2 cm from the bottom. After equilibration for 10 min above the solvent in a closed glass tank, the strips were immersed to a depth of 1 cm. After three ascents for a distance of 10 cm (HEILMANN *et al.*, 1957), with drying at 80° for 10 min between each ascent, the strips were sprayed with a 0·25% ninhydrin solution in acetone. The colour was developed for 10 min at 80° and the intensity of the bands was measured after soaking in an α-bromonaphthalene–paraffin oil mixture, using a Zeiss Extinktionsschreiber II. Six chromatograms were run from each Warburg vessel (including the blanks). Known amounts of pure amino acids were used as standards. Whenever identification of an amino acid gave rise to difficulties, use was made of other solvent systems such as *n*-butanol–95% ethanol–water (80 : 20 : 20 v/v), or 'collidine'–water (200 : 70 v/v) + diethylamine (DENT, 1948).

The influence of changes in concentration of some of the compounds on the inhibition of the oxygen consumption and the quantity of amino acid in the incubation fluid was also studied. The data obtained for chlorpromazine are summarized in Table 1; those for orphenadrine in Table 2.

In some additional experiments with orphenadrine as inhibitor, after incubation for 60 min, not only the supernatant fluid was chromatographed, but also the fluid obtained by homogenization of the sedimented brain tissue in a Potter-Elvehjem homogenizer in 2·5 ml of phosphate buffer with subsequent centrifugation. The same operation was applied to the corresponding blank experiments (Table 3).

RESULTS AND DISCUSSION

O_2-*consumption*

The literature describes many, more or less detailed investigations into the influence of chlorpromazine on the metabolism of isolated brain tissue (DESCI and MÉKES, 1959; HADNAGY *et al.*, 1958; MCILWAIN, 1957; QUASTEL *et al.*, 1957; STARBUCH and HEIM, 1959; STRECKER, 1958; and many others. See also BRÜCKE, 1959). Some work has also been devoted to the study of the effect of some other psychotropic drugs on metabolism (see the above authors, and KIRPEKAR and LEWIS, 1959).

All the investigations show an inhibition of aerobic metabolism by chlorpromazine which is dependent, on the concentration of the chlorpromazine. Among other factors, the effect of promethazine (QUASTEL *et al.*, 1957; 1958) on the oxygen consumption is similar to that of chlorpromazine. On the other hand, concentrations of from 2×10^{-5} to 2×10^{-4} M-reserpine (KIRPEKAR and LEWIS, 1959) do not inhibit the oxygen consumption; at a concentration of 7×10^{-4} M, slight inhibition is observed. Azacyclonol causes slight inhibition (BRÜCKE, 1959), but no significant inhibition by L.S.D. (10^{-4} M) has been observed (STARBUCH and HEIM, 1959). Finally, 2×10^{-4} M-benactyzine, 10^{-4} M-, captodiamine, and 10^{-3} M-meprobamate (DESCI and MÉKES, 1959) do not reduce the oxygen uptake. At the concentrations used, however, these compounds do cause an uncoupling of oxidative phosphorylation (DESCI and MÉKES, 1959), as has also been found for chlorpromazine (authors as above).

TABLE 1.—INFLUENCE OF VARIOUS CONCENTRATIONS OF CHLORPROMAZINE ON INHIBITION OF OXYGEN CONSUMPTION AND CONTENT OF GLUTAMIC ACID AND γ-AMINOBUTYRIC ACID IN INCUBATION FLUID

Chlorpromazine Concentration	Inhibition of O_2 consumption %	Glutamic acid μg/ml	GABA μg/ml
2×10^{-3} M	89	83	14
8×10^{-4} M	60	66	12
5×10^{-4} M	30	33	3
$1{\cdot}7 \times 10^{-4}$ M	30	8	2
1×10^{-4} M	29	8	1
$2{\cdot}4 \times 10^{-5}$ M	22	8	1
$1{\cdot}3 \times 10^{-5}$ M	16	8	1

TABLE 2.—INFLUENCE OF VARIOUS CONCENTRATIONS OF ORPHENADRINE ON INHIBITION OF OXYGEN CONSUMPTION AND CONTENT OF GLUTAMIC ACID AND γ-AMINOBUTYRIC ACID IN INCUBATION FLUID

Orphenadrine concentration	Inhibition of O_2 consumption %	Glutamic acid μg/ml	GABA μg/ml
3×10^{-3} M	83	83	13
2×10^{-3} M	60	83	13
$1{\cdot}5 \times 10^{-3}$ M	35	83	14
1×10^{-3} M	18	42	3
$7{\cdot}5 \times 10^{-4}$ M	10	33	2
5×10^{-4} M	10	8	1

TABLE 3.—DISTRIBUTION OF GLUTAMIC ACID AND γ-AMINOBUTYRIC ACID BETWEEN INCUBATION FLUID AND BRAIN TISSUE WITH AND WITHOUT ADDITION OF 0·002 M-ORPHENADRINE

	Incubation fluid		Homogenized brain tissue in 2·5 ml of buffer		Total	
	Glutamic acid μg/ml	GABA μg/ml	Glutamic acid μg/ml	GABA μg/ml	Glutamic acid μg/ml	GABA μg/ml
Control	18	1	115	13	133	14
	10	1	102	17	112	18
	9	1	99	12	108	13
Orphenadrine	115	14	29	2	144	16
	122	14	20	1	142	15
	110	6	17	1	127	7

The mean values for the inhibition of the oxygen consumption obtained with the compounds tested by us are given in Table 4. Nine phenothiazine derivatives were examined; promazine, at the concentration used (0·002 M), showed the strongest inhibition. However, owing to the low solubility of some of these compounds, they were not completely dissolved. It should also be remembered when dealing with tissue slices that 'concentration' is not a strictly defined term. Imipramine, which is structurally related to the phenothiazines, produced, like most of the latter compounds, a high percentage inhibition. Among the diphenylmethane derivatives, seven compounds were tested. These compounds produced a lower degree of inhibition with the exception of orphenadrine, which produced a degree of inhibition of oxygen consumption similar to that of the phenothiazines.

A further nine compounds, the structures of which showed little relationship, were examined. One of the noteworthy results obtained with these compounds was that deanol (β-dimethylaminoethanol), which can be considered as one of the 'alcohol' components of the ether orphenadrine, did not cause any inhibition.

In general it can be said that, with regard to compounds previously tested, our results correspond qualitatively with those given in the literature. Owing to differences in experimental conditions, however, a quantitative comparison is not feasible; nor, for the same reason, are the data given by different authors strictly comparable. The effect of all the compounds on oxidative phosphorylation is being investigated.

VOL.
5
1959/6

Amino-acid metabolism

The influence of the compounds tested on amino acid metabolism is, in our opinion, more remarkable. Various compounds produced very distinct changes in the amino acids found in the incubation fluid. The chromatograms, in particular, showed two distinct bands. In a previous communication by one of us (DE WAART, 1958) these bands were identified as methionine and glutamic acid. The present work, however, showed that they were γ-aminobutyric acid (GABA) and glutamic acid.

Another band is given by aspartic acid, which is pronounced in the chromatograms of the incubation fluid from experiments with a number of psychotropic drugs when compared with the blanks. Furthermore a band corresponding to alanine could be identified, which was, however, in most experiments not stronger than that given by the blanks. A chromatogram obtained with orphenadrine, together with the extinction curve, and the corresponding results of a blank experiment are compared in Fig. 1. Some minor bands in the chromatogram have not yet been identified. One of them may possibly be due to one or more peptides. It is hoped that a planned investigation, in which high tension electrophoresis will be used, will yield a more distinct separation of the bands.

A study of the influence of the compounds investigated on the amino acids in the incubation fluid (Table 4), shows that the psychotropic drugs fall into two categories; one showing a pronounced effect, the other hardly any. The former include most of the phenothiazines as well as imipramine and orphenadrine.

Although there appears to be a correlation between inhibition of the oxygen consumption and the appearance of larger quantities of glutamic acid and γ-aminobutyric acid in the incubation fluid (Table 4), a closer comparison between, for instance, chlorpromazine, orphenadrine and L.S.D. shows that this is not the case. Presumably we are concerned here with two different aspects of the mechanism of

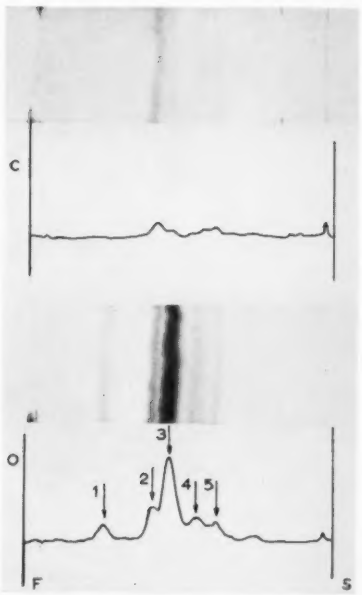


FIG. 1.—Paper chromatograms and corresponding absorption curves of amino acids (ninhydrin colouring) in incubation fluid of rat-brain slices after metabolizing aerobically for 1 hr.

(C) control experiment (without drug)
(O) 0·002 M-orphenadrine added
S = start, F = finish
Band 1 : γ-aminobutyric acid
Band 2 : alanine
Band 3 : glutamic acid
Band 4 : aspartic acid
Band 5 : one or more peptides (?)

VOL.
5
1959/6

TABLE 4.—INFLUENCE OF PSYCHOTROPIC DRUGS ON THE OXYGEN CONSUMPTION OF RAT-BRAIN SLICES AND CONTENT OF GLUTAMIC ACID AND γ-AMINOBUTYRIC ACID OF THE INCUBATION FLUID

Drug	Inhibition of O_2 consumption† %	Glutamic acid μg/ml‡	GABA μg/ml‡
Control		8	1
Phenothiazine derivatives			
Promazine	92	76	16
Chlorpromazine	89	91	15
Prochlorperazine*	46	50	3
Perphenazine*	33	8	1
Thiopropazate	40	17	2
Promethazine	84	83	14
Thiazinaminum	66	125	40
Diethazine	72	125	24
Ethopropazine*	25	8	6
Iminodibenzylderivative			
Imipramine*	75	66	9
Diphenylmethanederivatives			
Diphenhydramine	28	14	3
Orphenadrine	59	91	12
Captodiamine*	28	8	1
Hydroxyzine*	43	75	12
Benactyzine	25	25	1
Pipradol	22	8	1
Azacyclonol	29	25	1
Miscellaneous Compounds			
Reserpine*	8	8	1
L.S.D.	49	8	1
Adrenochrome*	0	8	1
Mescaline	8	25	4
Meprobamate	14	8	1
Phenaglycodol	30	8	1
Ectylurea*	0	8	1
Deanol	0	8	1
Iproniazide	4	8	1

* No clear solution at 0·002 M.

† The values are the means of a few sets of determinations. With chlorpromazine and orphenadrine, the much larger number of experiments permitted the calculation of standard deviations. (Chlorpromazine, 89 ± 2·8% for 11 experiments; orphenadrine, 59 ± 3·5% for 17 experiments).

‡ Means calculated from a few determinations except for orphenadrine. The necessarily somewhat more inaccurate evaluation of the chromatographic results is reflected in the standard deviations. (In 42 experiments with orphenadrine: glutamic acid, 91 ± 13·2 μg/ml; γ-aminobutyric acid, 12 ± 4·5 μg/ml. In 36 control experiments: glutamic acid 8 ± 4·3 μg/ml; γ-aminobutyric acid, 1 ± 0·4 μg/ml.

action of the compounds in question: an effect on certain phases of the respiratory chain and an effect of the membrane permeability. The experiments, the results of which are summarised in Table 3, distinctly show this latter effect. The glutamic acid and the γ-aminobutyric acid, which are found in the incubation fluid under the influence of orphenadrine, are regained by the brain tissue in the blank experiments. The total quantity of these amino acids, extracellular plus intracellular, is not apparently altered by orphenadrine. This is in agreement with the observation of ROBERTS and BAXTER (1959) that the γ-aminobutyric acid content of the brain of white mice remained unchanged upon administration of a large variety of drugs. Our observations, however, indicate that, although the total quantity of the amino acids in question may not change, a number of psychotropically active compounds are able to reduce the capacity of isolated brain tissue to regain these amino acids after the initial release.

Any extrapolation of our observations *in vitro* to the situation in the intact organism would clearly be premature. However, as a balance between glutamate and γ-aminobutyric acid in the free or occluded forms may to some degree determine neuronal activity in the brain, and as the factors which affect the absorption (occlusion) of the two substances may determine the actual balance of their activity (ELLIOT and VAN GELDER, 1958), we intend to continue our investigations in this direction, especially with regard to a possible relationship with sodium–potassium shifts.

VOL.
5
1959/6

SUMMARY

Using standard Warburg techniques the influence of several psychotropic drugs on the oxygen consumption of metabolizing rat brain slices was investigated. Depending on the chemical structure of the drugs, oxygen consumption was inhibited from 0 to 92% under the experimental conditions used.

Several of the drugs, mainly phenothiazines, imipramine and orphenadrine influence the distribution of amino acids (glutamic acid and γ-aminobutyric acid) between the brain slices and the incubation fluid.

REFERENCES

BRÜCKE F. (Editor) (1959) *Biochemistry of the Nervous System.* Pergamon Press, London.

DESCI L. and MÉKES J. (1959) *Arch. int. Pharmacodyn.* **119,** 294.

DENT C. E. (1948) *Biochem. J.* **43,** 169.

ELLIOT K. A. C. and VAN GELDER N. M. (1958) *J. Neurochem.* **3,** 28.

HADNAGY CS., EPERJESSY A., KISS A., CSEGEDY J., DÉZSI Z., HANTZ A. and ERDEI P. (1958) *Arch. int. Pharmacodyn.* **117,** 395.

HEILMANN J., BARROLLIER J. and WATZKE E. (1957) *Hoppe-Seyl. Z.* **309,** 219.

KIRPEKAR S. M. and LEWIS J. J. (1959) *Brit. J. Pharmacol.* **14,** 40.

MCILWAIN H. (1957) *Biochemistry and the Central Nervous System.* Churchill, London.

MCILWAIN H. (1959) In *Biochemistry of the Nervous System* (Edited by BRÜCKE F.) Vol. 3, pp. 46, 58, Pergamon Press, London.

NAUTA W. TH., OOSTERHUIS H. K., DE WAART C. and ERNSTING M. J. E. (1958) *Lancet* 591.

NAUTA W. TH. (1959) *Biochemistry of the Nervous System* (Edited by BRÜCKE F.) Vol. 3, p. 278. Pergamon Press, London.

PFEIFFER C. C. (1959) *Neurology (Minneapolis)* **9,** 249.

QUASTEL J. H., LINDEN O. and SVED S. (1957) *Canad. J. Biochem. Physiol.* **35,** 1135, 1145.

QUASTEL J. H., LINDEN O. and SVED S. (1958) In *Psychopharmacol.* p. 1. Hoeber, New York.

ROBERTS E. and BAXTER C. F. (1959) *Biochemistry of the Nervous System* (Edited by BRÜCKE F.) Vol. 3. p. 268. Pergamon Press, London.

ROBITSCHER L. B. (1958) *Amer. J. Physiol.* **114,** 1113.
SCHEURLE G. (1957) *Dtsch. med Wschr.* **82,** 2227.
STARBUCH W. C. and HEIM H. C. (1959) *J. Amer. pharm. Ass.* **48,** 251.
STRECKER H. J. (1958) In *Psychopharmacology*, Hoeber, New York, p. 23.
DE WAART C. (1956) *Acta physiol. pharm. neerl.* **5,** 220.
DE WAART C. (1958) *Some derivatives of diphenhydramine as inhibitors of the intermediary metabolism.* Thesis, Vrije Universiteit, Amsterdam.

OL.
5
9/60

Journal of Neurochemistry 1960, Vol. 5, pp. 128–134. Pergamon Press Ltd. Printed in Northern Ireland

EFFECT OF 5-HYDROXYTRYPTOPHAN AND SEROTONIN ON GLUCOSE CONTENT AND FUNCTIONAL ACTIVITY OF THE PERFUSED CAT BRAIN

J. MAGNES and SARAH HESTRIN-LERNER
Rogoff Laboratory of Physiology, Hebrew University
Hadassah Medical School, Jerusalem, Israel.

(*Received* 10 *September* 1959)

VOL.
5
1959/6

SINCE the demonstration by GADDUM (1953) that the induction of smooth muscle contraction *in vitro* by serotonin (5HT) may be inhibited by the potent hallucinogenic agent lysergic acid diethylamide, keen interest has been aroused in possible roles for serotonin in the central nervous system. Later, it was shown that 5HT is a natural component of brain tissue (TWAROG and PAGE, 1953) and that its precursor 5-hydroxytryptophan (5HTP), readily penetrates into the brain (UDENFRIEND, WEISSBACH and BOGDANSKI, 1957). In addition, it is known that the decarboxylase which transforms 5HTP to 5HT (GADDUM and GIARMAN, 1956) and an enzyme which is capable of oxidatively deaminating 5HT, are also present in brain tissue (SJOERDSMA, SMITH, STEVENSON and UDENFRIEND, 1955). Furthermore, it has been demonstrated that the tranquilizing agent reserpine, depletes the brain and other tissues of their serotonin content (SHORE, SILVER and BRODIE, 1955*a*).

There are numerous accounts of the central effects produced by the administration to laboratory animals of 5HT and 5HTP, but only one deals with cerebral metabolism *in vivo*. CAHN, HEROLD, DUBRASQUET and BURET (1957) report that in rabbits cerebral glucose consumption is reduced after giving 5HT intravenously.

In previous reports from this laboratory, it was shown that after an hour's perfusion with 'simplified blood' cat brain cortex becomes practically devoid of reducing substances (GEIGER, MAGNES, TAYLOR and VERALLI, 1954; ALLWEIS and MAGNES, 1958). The present study demonstrates that when 5HTP or 5HT is added to the perfusing fluid, the glucose content of the brain is restored to approximately the same level found in non-perfused cats, anaesthetized with nembutal.

The effects of 5HTP and 5HT on the functional activity and the electrocorticogram of the perfused cat brain, are also presented.

METHODS

Isolation of the cerebral circulation in cats was performed under nembutal anaesthesia according to the method of GEIGER and MAGNES (1947). Perfusion of the brain at a constant rate of blood flow without recirculation was carried out as described by ALLWEIS and MAGNES (1958). Cerebral blood flow was set so as to give 30 ml/min, which is equivalent to 100–140 ml/100 gm brain per min (depending on the weight of the brain). Variations in cerebral vascular resistance were recorded by means of a mercury manometer, connected to the arterial side of the perfusion circuit.

The simplified perfusion blood, consisting of exaustively washed bovine red cells suspended in Krebs-Ringer solution, was prepared as described previously (ALLWEIS and MAGNES, 1958). Respiration was recorded from a T-tube in the trachea and peripheral arterial pressure from a femoral artery. Cerebral A-V oxygen differences were recorded continuously with the photometric device

described by GEIGER and MAGNES (1947). The cerebral venous blood was collected throughout the experiments in NaF-coated cylinders immersed in an ice bath. Each cylinder received the venous outflow of an 8 min period. Owing to the virtually constant composition of the arterial blood over a period of one hr, it sufficed to take arterial samples at the beginning and end of each experiment. Glucose, lactate, Na and K were determined in all blood samples. At the end of each experiment the skull was trephined and the cerebral hemispheres were widely exposed. Brain cortex samples were excised with a scalpel while the circulation was still intact, and rapidly transferred to frozen CO_2. The tissue was then analysed for glucose, glycogen, lactate, Na and K.

The functional activity of the brain was estimated continuously by testing the corneal reflex and sensitivity to sound (click stimuli) and by observing the spontaneous movements of the animal and recording respiration and peripheral blood pressure. Functional activity was graded as excellent, good, poor or absent. Electrocortical activity was recorded from the frontal and parietal areas by means of electrodes inserted through burr-holes in the skull.

Serotonin or 5HTP (*) were added to the perfusing blood, just before it entered the carotid arteries, by means of a constant rate injection apparatus. Solutions of the compounds were infused at a standard rate of 1 ml/min over an 8 min period between the 24th and 32nd minutes of perfusion. The concentration of the injected substances varied between 5 and 40 μg per min for 5HT, and between 4·5 and 5·5 mg/min for 5HTP. When higher concentrations of 5HT were used severe cerebral vasoconstriction developed which prevented continuation of perfusion.

After precipitation of serum and brain samples with $Ba(OH)_2$ and $ZnSO_4$ the following analytical procedures were employed: SOMOGYI's (1952) iodometric method for serum glucose, NELSON's (1944) colorimetric modification for brain glucose, and the method of BARKER and SUMMERSON (1941) for brain and serum lactate. Brain glycogen was determined according to KERR (1936), and serum and brain Na and K by flame photometry.

RESULTS

Metabolic

The values for glucose, glycogen and lactate in cat brain cortex after one hour's perfusion with 'simplified blood' with and without the addition of 5HTP or 5HT, are presented in Table 1. Only experiments in which functional activity was excellent or good are included. This table shows that the addition of 5HTP or 5HT to the perfusion blood caused a significant, though variable, increase in the glucose content of the brain in comparison with the untreated controls. The glycogen content did not seem to be affected. It will also be noted that in the group receiving 5HTP the lactate content of the cortex was on the average lower than in the other experiments of this series. When cerebral functional activity was poor or failing neither 5HT or 5HTP had any effect on brain glucose values,

In order to determine whether the rise in glucose content, after the administration of 5HTP or 5HT, could be accounted for by an increase in the rate of glucose uptake by the brain, the rate of uptake for each 8-min period of the experiments was calculated. As pointed out previously (ALLWEIS and MAGNES, 1958; GEIGER and MAGNES, 1947), glucose uptake fluctuates considerably during any individual perfusion experiment, and the mean rate of glucose uptake varies from one experiment to another. Similar variations were observed in the experiments in which 5HTP and 5HT had been administered and it was found that the range of variation was as previously reported. It is concluded that the increase in glucose content of the cortex in the presence of 5HTP or 5HT cannot be accounted for by an increase in glucose uptake by the brain.

Lactate liberation for each of the 8-min periods of the experiments was also calculated. These calculations suggest that in comparison with control perfusion

* We are indebted to the Abbott Laboratories, Chicago, Ill. U.S.A. for a supply of 5-hydroxytryptophan and of serotonin creatinine sulphate.

experiments, lactate liberation during the period of injection was decreased by addition of 5HTP.

Oxygen uptake of the brain was not affected by the addition of 5HTP or 5HT, but remained steady and closely followed the pattern found in control perfusion experiments (GEIGER *et al.*, 1954; ALLWEIS and MAGNES, 1958).

TABLE 1.—GLUCOSE, GLYCOGEN AND LACTATE CONTENT OF CAT BRAIN CORTEX AFTER ONE HOUR OF PERFUSION. EFFECT OF 5HTP AND 5HT

Expt.	Total amount injected (*)	Brain content after 1 hr of perfusion mg/100 gm brain		
		Glucose	Glycogen	Lactate
103	none	7	21	153
104	none	5	32	67
107	none	9	73	103
108	none	21	21	124
201	25 mg 5HTP	61	70	50
202	36 ,, ,,	70	60	28
203	40 ,, ,,	43	9	121
204	40 ,, ,,	43	53	86
205	44 ,, ,,	37	34	104
301	300 μg 5HT	42	19	182
302	70 ,, ,,	51	28	107
303	40 ,, ,,	56	58	171
304	65 ,, ,,	30	54	122
305	50 ,, ,,	42	22	90
306	60 ,, ,,	32	3	125

* All injections were made at the rate of 1 ml/min over an 8 min period between the 24th and 32nd minutes of perfusion.

Na *and* K *content of brain*

Table 2 gives figures for the Na and K content of the brain at the end of an hour's perfusion with and without the addition of 5HTP or 5HT. Figures for control cats anaesthetized with nembutal, but not perfused, are also included. It will be noted that the Na content varied between 111 and 150 mg per cent and K between 310 and 490 mg per cent. In the perfusion experiments, the Na content of the 'simplified serum' was 140 m-equiv/l. and that of K was 5 m-equiv/l. In the non-perfused animals the Na content of the serum varied between 140 and 150 m-equiv/l. and the K content between 3 and 6 m-equiv/l. When compared with the tables compiled by FOLCH and LEBARON (1957) for mammalian cerebral grey matter, some of the K values in our experiments are on the high side, whereas the Na values are within the range given by these authors.

Functional and electrocortical activity

In a typical control perfusion experiment with 'simplified blood', functional activity is markedly improved and the electrocorticogram becomes desynchronized after the

first few minutes of perfusion. During this period most of the nembutal in the brain is probably washed out by the perfusion blood. The injection of 5HTP between the 24th and 32nd minutes of perfusion produced a reduction in functional activity and significant changes in the electrocorticogram. This was characterized by an increase in amplitude and drop in frequency of the waves, and by fluctuations in the baseline.

TABLE 2.—THE Na AND K CONTENT OF CAT CEREBRAL CORTEX
(mg/100 gm brain)

Expt.	Na	K
	Controls (not perfused)	
1	131	324
2	125	330
3	142	310
4	134	355
5	126	330
6	124	490
7	125	450
	Perfused (No Additions)	
105	111	344
106	146	368
107	150	400
108	135	378
	Perfused (5HTP added)	
201	145	390
202	130	430
203	124	400
205	150	450
	Perfused (5HT added)	
301	150	300
302	140	365
303	115	370
304	125	320
305	135	335

Towards the end of the experiment these changes became even more marked (Fig. 1). With 5HT the electrocorticographic pattern was less affected and high frequency–low amplitude activity frequently persisted despite a lowering of functional activity (Fig. 2).

DISCUSSION

It will be recalled that in control perfusion experiments with 'simplified blood' it was shown that the brain is unable to retain glucose in normal quantity (GEIGER *et al.*, 1954; ALLWEIS and MAGNES, 1958) and that a large fraction of the glucose taken up from the blood is transformed to lactate. The present experiments show that the administration of 5HTP or 5HT brings the brain glucose back to approximately the same level found in cats anaesthetized with nembutal, but otherwise intact, i.e. 40–60 mg per cent when the blood glucose is approximately 100 mg per cent (MAGNES,

unpublished results). Thus, it is suggested that perfusion with 'simplified blood' rapidly washes out the brain serotonin, with the result that at the end of an hour's perfusion, the brain is practically devoid of glucose. Obviously, brain serotonin determinations are indicated in future experiments.

It was pertinent to enquire whether increased glucose uptake from the blood, decreased oxidation of glucose, or decreased glycolysis was responsible for the increased glucose content of the cortex. In our experiments the rate of glucose uptake

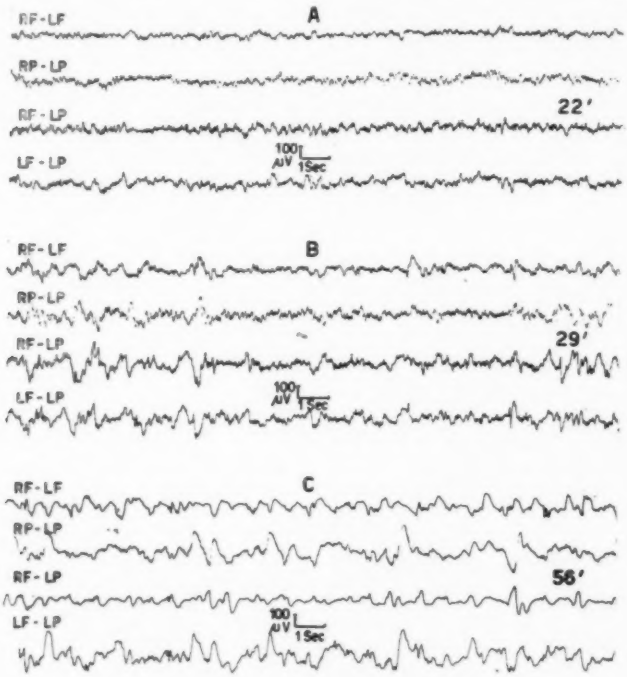


FIG. 1.—Effect of 5HTP on electrocortical activity of perfused cat brain. 44 mg of 5HTP were added to the perfusion blood between the 24th and 32nd minutes of perfusion.
A—22 min of perfusion; B—29 min of perfusion (while infusing 5HTP)
C—56 min of perfusion.
LF—Left frontal; RF—Right frontal;
LP—Left Parietal; RP—Right Parietal.

by the brain was not increased, nor was O_2 uptake affected by 5HTP or 5HT. CAHN *et al.* (1957) found that 5HT decreases cerebral glucose uptake in rabbits. On the other hand, there are indications that 5HTP depressed aerobic glycolysis to a certain extent since the lactate content of the brain was lower on the average and lactate liberation into the blood decreased during the period of 5HTP infusion. In control perfusion experiments, brain lactate was higher, and during the parallel period lactate liberation was still on the increase. It should be noted, however, that depression of glycolysis was not observed with 5HT.

Both 5HT and 5HTP caused a decrease in functional activity but, whereas 5HTP produced significant changes in electrocortical activity, 5HT had negligible effects. PIERRE and CAHN (1955) reported sedation in rats and rabbits given 5HT and the same

was found in mice by SHORE *et al.* (1955*b*). MONNIER and GANGLOFF (1957) have reported relaxation, drowsiness and changed EEG patterns after giving 5HTP intravenously to rabbits, and BRADLEY (1958) has demonstrated behavioural changes in conscious cats in the direction of general quietening and lessened motor activity. BOGDANSKI, WEISSBACH and UDENFRIEND (1958), have reported that low doses of 5HTP administered to dogs and cats caused a decrease in the spontaneous activity of these animals. In our experiments 5HT was, in most respects, less effective than 5HTP.

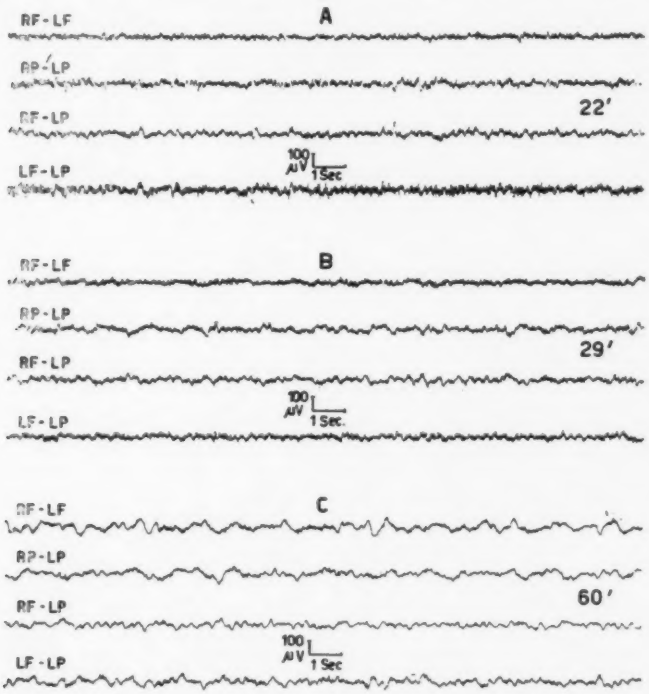


FIG. 2.—Effect of 5HT on electrocortical activity of perfused cat brain. 40 µg of 5HT were added to the perfusion blood between the 24th and 32nd minutes of perfusion.
A—22 min of perfusion; B—29 min of perfusion (while infusing 5HT).
C—60 min of perfusion.
LF—Left frontal; RF—right frontal;
LP—Left parietal; RP—right parietal.

This may be a reflection of the small amounts of 5HT actually available to the brain, owing to limited permeability of the brain to this substance (UDENFRIEND *et al.*, 1957) and to the relatively minute quantities administered.

With regard to the more general implications of the present experiments it will be recalled that in an earlier study (GEIGER *et al.*, 1954) it was demonstrated that many of the metabolic and functional deficiencies which are brought about by perfusing the brain with 'simplified blood' may be corrected by inserting an isolated liver into the perfusion circuit. The present study indicates that 5HTP and 5HT are able to correct one of the deficiencies, namely the inability of the perfused brain to maintain a certain store of glucose. Functional activity, on the other hand, does not seem to be improved

but rather is depressed. The relation of our results to those of GEIGER and YAMASAKI (1956) who found that the addition of uridine and cytidine to simplified blood maintains the brain in good functional condition with a normal carbohydrate metabolism for 4 hours or longer, remains to be clarified. The fact that the effects of 5HT and 5HTP were only present when functional activity of the brain was good or excellent requires further study. In the meantime it is tempting to speculate on the possibility that serotonin is involved in the regulation of carbohydrate metabolism in the central nervous system, and possibly in other animal organs, as well as in plants.

SUMMARY

Glucose content of cat cerebral cortex was higher in perfusion experiments when 5HTP or 5HT were added to the 'simplified blood' than in control perfusions without these additions. Neither glucose uptake nor oxygen consumption were affected whereas there are some indications that 5HTP tends to depress aerobic glycolysis. Both these substances depress functional activity of the brain. 5HTP caused electrocortical activity to shift from a high frequency–low amplitude pattern to a lower frequency and higher amplitude, while 5HT had negligible effects.

Acknowledgements—The assistance of D. BEN-SHACHAR, S. BEN-YONAH, R. FARKAS, L. KATZ and L. TALVI is gratefully acknowledged.

REFERENCES

ALLWEIS C. and MAGNES J. (1958) *J. Neurochem.* **2,** 326.
BARKER S. B. and SUMMERSON W. H. (1941) *J. biol. Chem.* **138,** 535.
BOGDANSKI D. F., WEISSBACH H. and UDENFRIEND S. (1958) *J. Pharmacol.* **122,** 182.
BRADLEY P. B. (1958) In *5-Hydroxytryptamine* (Edited by LEWIS G. P.), p. 214. Pergamon Press, London.
CAHN J., HEROLD M., DUBRASQUET M. and BURET J. P. (1957) *C. R. Soc. Biol., Paris* **151,** 82.
FOLCH-PI J. and LEBARON F. N. (1957) In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 67. Pergamon Press, London.
GADDUM J. H. (1953) *J. Physiol.* **121,** 15 P.
GADDUM J. H. and GIARMAN N. (1956) *Brit. J. Pharmacol.* **11,** 88.
GEIGER A. and MAGNES J. (1947) *Amer. J. Physiol.* **147,** 517.
GEIGER A., MAGNES J., TAYLOR R. N. and VERALLI M. (1954) *Amer. J. Physiol.* **177,** 138.
GEIGER A. and YAMASAKI S. (1956) *J. Neurochem.* **1,** 93.
KERR S. E. (1936) *J. Biol. Chem.* **116,** 1.
MONNIER M. and GANGLOFF H. (1957) *Helv. physiol. Acta* **15,** 83.
NELSON N. (1944) *J. biol. Chem.* **153,** 375.
PIERRE R. and CAHN J. (1955) *C. R. Soc. Biol., Paris* **149,** 1406.
SHORE P. A., SILVER S. L. and BRODIE B. B. (1955*a*) *Science* **122,** 284.
SHORE P. A., SILVER S. L. and BRODIE B. B. (1955*b*) *Experientia* **11,** 272.
SJOERDSMA A., SMITH T. E., STEVENSON T. D. and UDENFRIEND S. (1955) *Proc. Soc. Exp. Biol., N.Y.* **89,** 36.
SOMOGYI M. (1952) *J. biol. Chem.* **195,** 19.
TWAROG B. M. and PAGE J. H. (1953) *Amer. J. Physiol.* **175,** 157.
UDENFRIEND S., WEISSBACH H. and BOGDANSKI D. F. (1957) *Ann. N. Y. Acad. Sci.* **66,** 602.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 135–144. Pergamon Press Ltd. Printed in Northern Ireland

THE ACTION OF LYSOLECITHIN AND OF SNAKE VENOM ON WHOLE-CELL PREPARATIONS OF BRAIN, MUSCLE AND LIVER

B. McArdle,* R. H. S. Thompson and G. R. Webster
Department of Chemical Pathology, Guy's Hospital Medical School, London, S.E.1.

(*Received* 28 *September* 1959)

OL.
5
9/60

The action of lysolecithin and of snake venoms on the nervous system are of interest in two connections. Firstly, many of the neurotoxic venoms contain an active phospholipase A, and introduction of cobra venom, previously heated to inactivate enzymes other than phospholipase A, into the spinal cord of rats causes paralysis (Sanders, Akin and Soret, 1954; see also Quastel, 1958). Secondly, the work of Morrison and Zamecnik (1950) and of Birkmayer and Neumayer (1957) indicates that under *in vitro* conditions both phospholipase A and lysolecithin can cause demyelinating changes in the central nervous system; the possible participation of lysolecithin or some similar lytic substance in the aetiology of the plaques of multiple sclerosis has therefore been suggested.

Petrushka, Quastel and Scholefield (1959) have recently shown that when slices of rat-brain cortex are exposed to cobra-venom phospholipase A in the presence of pyruvate there is an initial increase in oxygen consumption, followed after a lapse of about 60 minutes by a pronounced fall; rat liver or kidney slices on the other hand show no such inhibition of oxygen uptake. Both lysolecithin and phospholipase A, however, are known to attack isolated liver mitochondria, depressing their rate of oxygen uptake and of oxidative phosphorylation (Braganca and Quastel, 1953; Nygaard and Sumner, 1953; Nygaard, 1953; Edwards and Ball, 1954; Nygaard, Dianzani and Bahr, 1954; Tookey and Balls, 1956; Witter, Morrison and Shepardson, 1957; Petrushka *et al.*, 1959).

The inhibition by cobra-venom phospholipase A of the respiration of brain slices and the apparent insensitivity of liver and kidney slices to the action of this enzyme would seem therefore to indicate either a more rapid and extensive action of phospholipase A on brain slices (resulting in a more rapid and complete penetration of the phospholipase A to the mitochondria), or that the respiratory processes of brain cells are more intimately dependent on the integrity of the cell membrane than is the respiration of liver or kidney cells.

Therefore it seemed desirable to study the action of lysolecithin and of snake venoms containing phospholipase A on whole-cell preparations of brain and liver, in order to determine whether there are differences in the action of these lytic agents on the cell membranes of these two tissues, as distinct from the mitochondrial membranes. We have also studied the action of these two substances on muscle, using the rat

* External Scientific Staff, Medical Research Council.

diaphragm, since alterations of muscle-cell membranes (as demonstrated by release of aldolase and glutamic–oxaloacetic transaminase into the tissue fluids) may play a part in various types of myopathy.

It has recently been shown that incubation of brain slices with lysolecithin for 30 minutes causes a rapid release of both true and pseudocholinesterase from the slices into the ambient fluid (Marples, Thompson and Webster, 1959). In the present work we have measured the rate of outflow of glutamic–oxaloacetic transaminase from slices of brain or liver, or from segments of rat diaphragm, incubated in the presence and absence of lysolecithin or of snake venom, as an initial criterion for the detection of gross effects on cell membranes induced by these compounds. Our reasons for choosing this enzyme were: (i) the level of its activity in brain, muscle and liver is high (Cohen and Hekhuis, 1941), (ii) in the liver cell it is known that the greater part of it is contained in the 'cell sap' (Müller and Leuthardt, 1950; Hird and Rowsell, 1950; Schneider, 1956), and (iii) it has been shown that it is not inhibited by lysolecithin (Marples *et al.*, 1959).

In most of the experiments described in this paper we have used the unheated lyophilized venom. However, in order, to relate the observed effects, even though only provisionally, to the action of the phospholipase A contained in the venom, we have also carried out experiments with solutions of venom heated to 100° for 10 min to inactivate enzymes other than the relatively heat-stable phospholipase A.

In a few experiments we have made further observations on the release from brain slices of the true cholinesterase. Although satisfactory evidence as to the intracellular localization of this enzyme in brain has until recently been meagre, there were indications, both experimental and based on *a priori* physiological considerations, that part at any rate of this enzyme is located in the cell membrane. Aldridge and Johnson (1959) have, however, recently carried out a careful study of the distribution of true cholinesterase in subcellular fractions of rat brain, and have shown that 35–40 per cent of the enzyme is sedimented in the microsomal fraction. Nathan and Aprison (1955) had earlier reported a relatively high concentration of cholinesterase in the microsome fraction of rabbit brain. Toschi (1959) and Hanzon and Toschi (1959), using both biochemical techniques and electron microscopy, have studied the brain microsome fraction in detail; they too found the highest concentration of cholinesterase to occur in this fraction, and showed that it is mainly associated with the membranous structures rather than with the particles that make up most of the microsome fraction.

We have in addition measured, over 3 successive 20 min periods, the oxygen consumption of the brain or liver slices, or of the diaphragm segments, respiring in a glucose-containing medium in the presence and in the absence of lysolecithin or snake venom.

A further reason for studying the influence of these compounds on the release of intracellular enzymes such as glutamic–oxaloacetic transaminase was the hope that measurement of the release of such enzymes might provide a convenient and rapid method for detecting the presence in biological fluids of substances capable of exerting a lytic action on cells of the nervous system; claims for the existence of such substances in the cerebrospinal fluid, plasma or urine of patients with multiple sclerosis have been made by Weil, Luhan and Balser (1935), Weil and Heilbrunn (1941), Birkmayer and Neumayer (1957) and Wolfgram and Rose (1958). The methods

VOL.
5
1959/6

used by these workers for the detection of neurolytic substances have not, however, been entirely satisfactory, since they have in most cases involved long periods (up to 72 hr) of incubation of nervous tissue with the fluid under investigation, followed by histological staining for myelin.

EXPERIMENTAL

Tissue preparations. Slices of rat cerebrum or rat liver were cut free-hand with a safety-razor blade moistened with the buffer solution in which they were subsequently suspended. The slices were transferred from the blade to a Petri-dish containing 10–15 ml of the buffer.

In experiments with rat diaphragm the tissue was removed from the animal and placed in a Petri dish containing buffer in order to wash it free from adhering blood. The central tendinous portion, together with any fat or connective tissue attached to the parietal border, was dissected away, and the two hemi-diaphragms bisected radially.

Incubation procedure. The slices or quarter-diaphragms were placed, after draining excess fluid from them, in tared Warburg flasks containing 2·7 ml of saline buffer, pH 7·4 containing 0·011 M-glucose. Both the bicarbonate and the phosphate buffer mixtures (pH 7·4) described by DICKENS and GREVILLE (1935) have been used. The flasks were then re-weighed (weights of tissue added varied from 50 to 150 mg), and lysolecithin or venom, dissolved in the buffer being used, was added to the side-bulbs. With the bicarbonate buffer the vessels were gassed with a 95% O_2 + 5% CO_2 mixture. After temperature equilibration (38°) the lytic agent was tipped in from the side-bulbs, and the slices exposed to its action for periods varying from 10 to 120 min. When O_2 uptake was being measured (KOH papers in centre well), readings were taken at 0, 20, 40, 60 and 120 min.

After the incubation period the contents of the flasks were pipetted into tubes and centrifuged at 3000 rev/min (1800 **g**) for 2 min. The clear or almost clear supernatant solutions were then pipetted off for assay of glutamic–oxaloacetic transaminase (GOT) or, in the case of brain only, of true cholinesterase (ChE).

In all experiments duplicate flasks were set up for each concentration of the lytic agent, and duplicate enzyme assays carried out on the supernatants obtained from each of these flasks. Duplicate assays were also carried out on the fluid in which tissue had been incubated in the absence of any added lytic agent. Homogenates of fresh slices of brain or liver or of quarter diaphragms in the buffer mixture (about 100 mg tissue/30 ml) were also prepared in order to determine the total GOT activity of the tissue under investigation. For total true ChE determination 100 mg brain slices were homogenized in 6 ml buffer. By this means it was possible to express the activity found in the ambient fluid as a percentage of the total activity in the tissue.

Transaminase estimation. Activity was determined on 0·5 ml samples of the homogenates or of the supernatant fluids obtained from the Warburg flasks by centrifugation, after dilution of the latter 1 in 10 with distilled water. The colorimetric method of CABAUD, LEEPER and WROBLEWSKI (1956) was used, involving incubation of the sample under investigation with an aspartate–α–ketoglutarate reagent, followed by estimation of the oxaloacetate formed after conversion to pyruvate by an aniline citrate reagent. Activity is expressed as μmoles oxaloacetate formed per g per hr, or as a percentage of the total activity in the slice.

True cholinesterase was estimated manometrically with suitable blanks on 2 ml samples of the undiluted supernatant fluids obtained by centrifugation using 0·03 M-acetyl-β-methylcholine chloride (L. Light & Co. Ltd.) as substrate. Activity is expressed as μl CO_2/g per hr, or as a percentage of the total activity in the slice.

Lysolecithin. Prepared from ovolecithin; the latter was obtained from fresh eggs, and was purified by chromatography on an alumina column according to the method of RHODES and LEA (1957). Conversion to lysolecithin was brought about by the action of the dried venom of the Black Cottonmouth moccasin (see below) on a 1% solution of the purified lecithin in ether, using the method of LONG and PENNY (1957). Crystallization of the lysolecithin was carried out from hot ethanol as described by SAUNDERS and THOMAS (1958).

Phospholipase A. Freeze-dried venom of the Black Cottonmouth moccasin (*Agkistrodon piscivorus piscivorus*), obtained from Ross Allen's Reptile Institute, was used as the source of the enzyme.

RESULTS

Transaminase release from rat brain slices

The effects produced by three different concentrations of lysolecithin and one concentration of venom on the release of transaminase from rat-brain slices have been studied. The times of incubation with and without the lytic substances have ranged from 10 to 120 min, and at one incubation time we have compared a phosphate

Table 1.—Release of glutamic-oxaloacetic transaminase from rat-brain slices brought about by incubation with lysolecithin or venom

(Enzyme activity expressed as μmoles oxaloacetate formed/g tissue per hr. Number of experiments given in brackets.)
Mean activity of homogenate of brain slices = 2145 μmoles/g per hr (S.E.M. = 72, n = 22).

Incubation time (min)	Buffer	GOT activity present in incubation medium after exposure to:				
		Nil	Lysolecithin (mM)			Venom (167 μg/ml medium)
			1·2	6·0	12·0	
10	Phosphate	204 (2)	705 (1)	1390 (2)	—	755 (1)
20	Phosphate	326 (6)	—	—	1795 (3)	987 (3)
20	Bicarbonate	341 (8)	908 (4)	1395 (3)	1740 (7)	—
60	Phosphate	353 (4)	898 (2)	1593 (2)	1738 (1)	1437 (2)
120	Phosphate	390 (7)	—	—	—	1771 (4)
		Activity present in incubation medium expressed as percentage of mean activity in brain homongenate				
10	Phosphate	10	33	65	—	35
20	Phosphate	15	—	—	84	46
20	Bicarbonate	16	42	65	81	—
60	Phosphate	16	42	75	81	67
120	Phosphate	18	—	—	—	83

and a bicarbonate buffer mixture as the incubation medium. The results are summarized in Table 1. The activity released from the slices into the incubation medium during the period of incubation has been expressed in terms of μmoles oxaloacetate formed per g brain slices per hr, and also as a percentage of the mean GOT activity present in a homogenate of the slices. In 22 experiments the mean activity of a homogenate of slices was 2145 μmoles/g per hr.

It will be seen that in the absence of any added lytic agent about 10% of the total GOT activity originally present in the slices is released into the ambient fluid as a result of incubation for 10 min at 38° in the buffered glucose–saline mixture. If the incubation time is increased, the enzyme activity released undergoes a slow increase, rising to 18 per cent of the total activity of the slices after a 2-hr incubation period. It is of interest to recall that Stern, Eggleston, Hems and Krebs (1949),

in the course of their work on the factors influencing the accumulation of glutamate in isolated brain tissue, have also reported a substantial loss of GOT from slices of guinea pig brain cortex, 25 per cent of the enzyme passing from the slices into the medium after incubation for 40 min in glucose–saline.

Exposure of the slices to the 3 concentrations of lysolecithin which we have studied causes a very significant increase in the amount of transaminase released. The lysolecithin appears to exert its maximal effect within 10–20 min of its addition to the slices, incubation for 60 min causing very little, if any, increase in the amount

TABLE 2.—TRUE CHOLINESTERASE RELEASE FROM RAT-BRAIN SLICES DUE TO INCUBATION WITH LYSOLECITHIN AT 38°

(Slices suspended in $NaHCO_3$ buffer, pH 7·4 (DICKENS and GREVILLE, 1935). Results expressed as μl CO_2/g per hr. Number of experiments given in brackets.)
Mean activity of homogenate of slices = 9239 (S.E.M. = 445, n = 10).

Incubation time (min)	Activity present in incubation medium after exposure to:			
	Nil	Lysolecithin (mM)		
		1·2	6·0	12·0
20	194 (5)	1145 (2)	2708 (2)	4134 (5)
60	63 (1)	1028 (1)	3330 (1)	3905 (1)
	Activity expressed as percentage of mean homogenate activity			
20	2	12	29	45
60	1	11	36	42

released. Incubation of the slices in a bicarbonate buffer mixture instead of the phosphate mixture did not significantly influence the rate of release of the enzyme either in the presence or absence of added lysolecithin.

Table 1 also shows the amount of enzyme released by unheated moccasin venom (167 μg/ml of incubation medium); in this case the amount of enzyme released appears to increase with increasing times of incubation. Four further experiments were carried out with aqueous solutions of venom which had been heated for 10 min at 100°; in each case the addition of the heated venom caused a significant release of GOT, although the effect was smaller than with the unheated venom, the activity in the incubation medium varying from 34 to 78 per cent of that resulting from the presence of unheated venom.

True cholinesterase release from rat brain slices

In Table 2 are given the results of the true cholinesterase estimations in the homogenates and in the centrifuged ambient fluid following incubation for 20 min of slices with the same concentrations of lysolecithin. In the absence of any added lytic agent only 2 per cent of the total true cholinesterase activity of the slice was

released, a figure in marked contrast to the 15 per cent release of transaminase. In each case the added concentrations of lysolecithin caused a very significant increase in the amount liberated from the slices, although the highest concentration studied, 12×10^{-3} M, released only 45 per cent of the total activity.

Again, incubation of the slices for 60 min caused no further increase in cholinesterase release.

Table 3.—Comparison of glutamic-oxaloacetic transaminase from brain, muscle and liver after exposure to lysolecithin or venom

(Slices exposed for 60 min at 38° in phosphate buffer. Results expressed as mean values in μmoles oxaloacetate/g per hr. Number of experiments given in brackets.)

Tissue	Mean homogenate activity ± S.E.M.	Activity in supernatant in presence of:			
		Nil	Lysolecithin (mM)		167 μg Venom/ml
			1·2	6·0	
Brain	2145 ± 72 (n = 22)	353 (4)	898 (2)	1593 (2)	1437 (2)
Muscle	2815 ± 243 (n = 9)	789 (3)	1575 (2)	1916 (3)	1880 (2)
Liver	2259 ± 111 (n = 10)	663 (5)	1456 (2)	1903 (3)	1341 (2)
			Release expressed as % of homogenate activity		
Brain		16	42	75	67
Muscle		28	56	68	67
Liver		29	64	84	59
			'Extra' % release due to lytic agent		
Brain		—	26	59	51
Muscle		—	28	40	41
Liver		—	35	55	30

Comparison of GOT *release from brain, muscle and liver*

Table 3 summarizes experiments in which the release of GOT has been compared in brain, muscle and liver, following incubation at 38° for 60 min in the presence of both 1·2 and 6 mM-lysolecithin and 167 μg moccasin venom/ml. It is of interest that the amount of this enzyme released into the ambient fluid in the absence of any added lytic agent is significantly greater in the case of both muscle and liver (about 30 per cent of the total) than in the case of brain (about 15 per cent of the total). In the case of all three tissues, however, a very substantial increase in the amount of this enzyme liberated from the tissue cells is brought about by the presence of either lysolecithin or venom. With lysolecithin the percentage release is very similar

VOL. 5 1959/6

in the case of each of these three tissues, although in the case of the venom a smaller release would seem to be produced from liver than from the other two tissues.

Effect of lysolecithin and venom on O_2 uptake of brain, liver and muscle in the presence of glucose

In confirmation of the results reported by QUASTEL (1958) and by PETRUSHKA *et al.* (1959) moccasin venom produces a delayed but extensive inhibition of oxygen uptake by rat-brain slices incubated in a glucose-containing medium, while producing

TABLE 4.—EFFECT OF LYSOLECITHIN AND VENOM ON O_2 UPTAKE BY RAT BRAIN AND LIVER SLICES AND BY RAT QUARTER-DIAPHRAGMS IN PRESENCE OF GLUCOSE

(O_2 uptake expressed as μl O_2/g per hr. Number of experiments given in brackets.)

Tissue	Time (min)	μl O_2/g per hr			Effect (%)	
		Control	Lysolecithin (6 mM)	Venom (167 μg/ml)	Lysolecithin	Venom
Brain	0–20	1060 (9)	1008 (2)	1114 (3)	−5	+5
	20–40	1023 (9)	586 (2)	615 (3)	−43	−40
	40–60	1008 (8)	516 (2)	505 (3)	−49	−50
	60–120	1015 (3)	399 (3)	342 (2)	−61	−66
Liver	0–20	777 (9)	1117 (3)	841 (2)	+44	+8
	20–40	675 (9)	689 (3)	646 (2)	−2	−4
	40–60	663 (9)	607 (3)	635 (2)	−8	−4
	60–120	664 (2)	389 (2)	558 (2)	−42	−16
Muscle	0–20	582 (7)	571 (2)	737 (3)	−2	+21
	20–40	571 (7)	461 (2)	546 (3)	−19	−4
	40–60	481 (6)	420 (1)	460 (2)	−13	−4
	60–120	406 (2)	200 (2)	378 (2)	−51	−7

only a slight inhibition of O_2 uptake by liver slices during the time studied (Table 4). We have further shown that under these conditions the effect on muscle (diaphragm) is also small, although there is a considerable stimulation of uptake (+21 per cent) in the first 20 min period.

Lysolecithin (6 mM) also causes a profound, progressive inhibition of respiration of brain slices. With liver, on the other hand, there is an initial marked stimulation (+44 per cent) during the first 20 min period, while, unlike brain, marked inhibition does not occur until after 60 min. Although muscle tissue shows some inhibition during the period 20–40 min, marked inhibition, comparable to that produced in the brain slices, again does not develop until after 60 min.

Intracellular localization of GOT *in brain*

Although the intracellular localization of GOT in liver cells has been described (MÜLLER and LEUTHARDT, 1950; HIRD and ROWSELL, 1950), we have been unable to find any report of the localization of this enzyme in brain. We have therefore carried out estimations of GOT activity in centrifuged fractions prepared from rat

brain by Dr. W. N. Aldridge and Mr. M. K. Johnson. The findings are shown in Table 5. It will be noted that the activities of the various fractions amount to 139 per cent of that of the whole homogenate. The reason for this apparent activation is not clear, and we are investigating this further. The supernatant fraction, however, contains the major proportion of the total activity.

Table 5.—GOT activity in centrifuged fractions prepared from rat brain

Activity of whole homogenate = 2985 μmoles/g per hr.

Fraction	Activity	Activity as % of whole homogenate
Nuclei	306	10
Heavy mitochondria	1175	39
Light mitochondria	393	13
Microsomes	382	13
Supernatant	1905	64
		139%

DISCUSSION

The results described above indicate that both lysolecithin and moccasin venom cause a marked increase in the amount of glutamic–oxaloacetic transaminase released from rat brain slices when incubated at 38° in buffered glucose–saline solutions.

This effect is a relatively rapid one, and, in the case of lysolecithin, appears to have reached a maximum within 10–20 min, no further increase in the amount of enzyme released occurring within the 60 min period studied. The amount of enzyme released by moccasin venom, on the other hand, increases with time.

As shown in Table 3, lysolecithin seems to exert this 'lytic' action, resulting in outflow of an intracellular protein, to a very similar extent on the cells of brain, muscle and liver. The effect of moccasin venom on cells of brain and muscle appears to be similar in magnitude under the conditions studied but it is possible that its effect on liver cells is less marked.

The results obtained with cholinesterase suggest that, as might be expected, wide differences exist in the magnitude of the release of different enzymes from the cell by lytic agents such as lysolecithin. It is of interest to note also that while about 15 per cent of the GOT is released from brain slices following incubation in the glucose–saline medium in the absence of any added lytic agent only 1–2 per cent of the true cholinesterase is released under comparable conditions. This relatively larger release of GOT from brain in the absence of lytic substances, noted earlier by Stern, Eggleston, Hems and Krebs (1949), is even more marked in the case of liver and muscle cells. The difference between the behaviour of GOT and cholinesterase is probably explicable in terms of their intracellular localization, since, while we have found that the major proportion of the GOT is present in the centrifuged supernatant of sucrose

VOL. 5 1959/6

homogenates of rat brain, ALDRIDGE and JOHNSON (1959) found that only 9 per cent of the cholinesterase was present in the supernatant, 35–40 per cent of it being in the microsomal fraction, and a further 40 per cent in the heavy and light mitochondrial fractions. Moreover, TOSCHI (1959) has concluded that there is a firm association of the cholinesterase with the membrane structures present in his microsomal fractions, on the grounds that there is a minimal removal of this enzyme from the microsome fraction after incubation with a number of lipolytic enzymes including phospholipase A.

We have not, as yet, studied heat-inactivated moccasin venom in any quantitative way, but have merely shown that a very substantial release of GOT from brain slices can be caused by aqueous solutions of venom heated for 10 min at 100°. Since such heated venoms had negligible, or no, proteolytic activity (hydrolysis of casein) we are provisionally concluding that the GOT release induced by the unheated venom is largely, if not entirely, due to the presence in the venom of phospholipase A. The fact that the effect produced by the heated venom amounted to only 34–78 per cent of that caused by the unheated venom may, we feel, be due to partial heat inactivation of the phospholipase A. Using copperhead venom, HUGHES (1935) has found that the phospholipase A is rapidly destroyed on boiling in solutions more alkaline than pH 7·0, although it is stable to prolonged boiling at pH 5·9, and in preliminary experiments with Dr. W. L. MAGEE we have found that the same is true for moccasin venom.

The extent of this rapid release of GOT is of interest when considered in relation to the effects produced by lysolecithin on the O_2 consumption of the slices. Thus, a concentration of lysolecithin (6×10^{-3} M) which produces in 10 min a total release of about 60 per cent of the GOT contained in the slice, or an amount of venom (167 μg/ml) which produces in 20 min a release of about 50 per cent of the total enzyme present, produces no effect on the O_2 consumption during that time, although in the period 20–40 min a profound inhibition of O_2 uptake occurs. A very extensive outflow of GOT from the cell can therefore occur without any simultaneous effect on the overall O_2 consumption of the tissue slices.

The effects of lysolecithin and of moccasin venom on the O_2 uptake of surviving cells differ widely as between brain, liver and muscle. We have confirmed the finding of QUASTEL (1958) that the respiration of brain slices is much more sensitive to inhibition by venom than is that of liver slices, and have further shown that rat diaphragm is also relatively insensitive. Respiration of brain cells seems also to be more sensitive to inhibition by lysolecithin, although with this compound a profound delayed inhibition of both liver and muscle manifests itself in the second hour. This lesser sensitivity of the respiration of muscle and liver to inhibition by these lytic agents, as compared with the marked and relatively rapid effect on brain, cannot be due to a lack of action of these agents on the cell membranes, since it has been shown that the transaminase release caused by venom is very similar in brain and muscle, while that due to lysolecithin is of the same order for all three tissues.

We do not propose, on our present evidence, to discuss the mechanism by which these lytic agents bring about this extensive release of GOT, but, by analogy with the use of the release of haemoglobin from erythrocytes as an index of haemolytic activity, we suggest that the measurement of the release of intracellular enzymes such as GOT from brain, muscle or liver cells may provide a simple and rapid method

for detecting the presence of substances capable of exerting a lytic action on these tissues, and for their semi-quantitative estimation.

SUMMARY

1. A study has been made of the action of lysolecithin and Black Cottonmouth moccasin venom on whole-cell preparations (slices) of rat brain and liver and on rat quarter-diaphragms.

2. Both lysolecithin and venom cause a rapid and striking release of glutamic–oxaloacetic transaminase from each of these tissues.

3. Lysolecithin has also been shown to cause a somewhat smaller release of true cholinesterase from rat brain slices.

4. In confirmation of results reported by Quastel (1958) it has been shown that the respiration of rat brain slices is more sensitive to inhibition by the venom than is the respiration of either liver or diaphragm. O_2 uptake by brain slices is also more rapidly inhibited by lysolecithin than is the case with muscle or liver.

5. The partition of GOT activity between the various subcellular fractions derived from rat brain has also been described.

Acknowledgements—Our thanks are due to Dr. W. N. Aldridge and Mr. M. K. Johnston for kindly providing the brain fractions, to Mr. A. A. Cernik for his technical assistance, and to the Medical Research Council for providing a grant towards the expense of this work.

REFERENCES

Aldridge W. N. and Johnson M. K. (1959) *Biochem. J.* **73,** 270.
Birkmayer W. and Neumayer E. (1957) *Dtsch. Z. Nervenheilk.* **177,** 117.
Braganca B. M. and Quastel J. H. (1953) *Biochem. J.* **53,** 88.
Cabaud P., Leeper R. and Wroblewski F. (1956) *Amer. J. clin. Path.* **26,** 1101.
Cohen P. P. and Hekhuis G. L. (1941) *J. biol. Chem.* **140,** 711.
Dickens F. and Greville G. D. (1935) *Biochem. J.* **29,** 1468.
Edwards S. W. and Ball E. G. (1954) *J. biol. Chem.* **209,** 619.
Hanzon V. and Toschi G. (1959) *Exp. Cell Res.* **16,** 256.
Hird F. J. R. and Rowsell E. V. (1950) *Nature, Lond.* **166,** 517.
Hughes A. (1935) *Biochem. J.* **29,** 437.
Long C. and Penny I. F. (1957) *Biochem. J.* **65,** 382.
Marples E. A., Thompson R. H. S. and Webster G. R. (1959) *J. Neurochem.* **4,** 62.
Morrison L. R. and Zamecnik P. C. (1950) *Arch. Neurol. Psychiat., Chicago* **63,** 367.
Müller A. F. and Leuthardt F. (1950) *Helv. chim. acta* **33,** 268.
Nathan P. and Aprison M. H. (1955) *Fed. Proc.* **14,** 106.
Nygaard A. P. (1953) *J. biol. Chem.* **204,** 655.
Nygaard A. P., Dianzani M. U. and Bahr G. R. (1954) *Exp. Cell Res.* **6,** 453.
Nygaard A. P. and Sumner J. B. (1953) *J. biol. Chem.* **200,** 723.
Petrushka E., Quastel J. H. and Scholefield P. G. (1959) *Canad. J. Biochem. Physiol.* **37,** 975.
Quastel J. H. (1958). Proc. IVth. Int. Congr. Biochem. p. 90. (Vienna, Sep. 1958).
Rhodes D. N. and Lea C. H. (1957) *Biochem. J.* **65,** 526.
Sanders M., Akin B. A. and Soret M. G. (1954) *Acta neuroveg.* **8,** 362.
Saunders L. and Thomas I. L. (1958) *J. Chem. Soc.* 483.
Schneider W. C. (1956) Proc. III rd. Int. Congr. Biochem. p. 305. (Brussels, Aug. 1955).
Stern J. R., Eggleston L. V., Hems R. and Krebs H. A. (1949) *Biochem. J.* **44,** 410.
Tookey H. L. and Balls A. K. (1956) *J. biol. Chem.* **220,** 15.
Toschi G. (1959) *Exp. Cell Res.* **16,** 232.
Weil A. and Heilbrunn G. (1941) *Proc. Soc. exp. Biol., N.Y.* **48,** 233.
Weil A., Luhan A. J. and Balser B. H. (1935) *Trans. Amer. neurol. Ass.* **61,** 142.
Witter R. F., Morrison A. and Shepardson G. R. (1957) *Biochim. et biophys. acta* **26,** 120.
Wolfgram F. and Rose A. S. (1958) *J. Neuropath.* **17,** 399.

VOL.
5
1959/6

trichloroacetic acid and the nitrogen content of the acid-insoluble material found by the micro-Kjeldahl method. The remainder of each extract was used for five or more paper electrophoretic separations of soluble protein, any material left over after four days being discarded. Freezing of the tissue before extraction, as recommended by HOFMANN and SCHINKO (1956) was not carried out as the degree of separation achieved was considered to be adequate. Freezing improves both the clarity of the electrophoretic pattern and the degree of extraction of the protein. Concentration of dilute extracts of the soluble proteins from brain tissue by dialysis against agar or dextran was not carried out, and undesirable changes in the protein pattern were brought about by this method. HAMMARSTON (1952) has reported that serum globulin is adsorbed on the walls of cellophane dialysis tubing during concentration of serum in dextran solution.

The electrophoretic separations were carried out in two perspex (acrylic resin) cabinets. Each was so constructed that eight paper strips, 5 cm by 30 cm, could be arranged in the same horizontal plane at the same height above the electrode compartments. The strips were individually stretched taut by shot-filled glass capsules in such a way that sag was negligible. The behaviour of chromatography paper as a supporting medium for the electrophoresis of the brain proteins was much improved by soaking it, for at least 12 hr, in the buffer before electrophoresis.

Paper strips, 5 cm in width and about 40 cm in length were cut from sheets of Whatman No. 2 chromatography paper. These strips were allowed to soak overnight or for at least 12 hr in the following buffer: 20·6 g of soluble barbitone (sodium diethyl barbituric acid) and 1·3 g of citric acid, in 2000 ml of distilled water. The components of the buffer dissolve rapidly if they are added separately. The pH of this buffer is 8·6 and the molarity, with respect to the sodium salt, 0·05 M. When the extracts of protein were ready for electrophoresis, the paper strips were taken out of the buffer and hung out of contact with one another in an enclosed space, to drain for about 20 min. After placing the strips in position in the apparatus, extract (0·1 ml) was applied on the edge of each of eight cover glasses to the eight strips in each cabinet, in a line about 5 cm from the negative end of the paper. The cover glasses were supported by leaning them against a rod running the width of the apparatus, and which was removed when the extracts had drained into the zone of application on the paper. A light spray of the cold buffer solution was applied to the paper from the positive end up to the point where the protein was applied, until the surface of the paper appeared wet. This helped to eliminate buffer flow from the ends of the paper towards the centre, thus preventing any disturbance of the position of the protein on the paper. When all the protein samples were applied, 500 ml of cold electrophoresis buffer were poured into each electrode compartment and the apparatus was closed with a tightly-fitting lid. Blotting paper on the underside of each lid prevented condensed moisture from dropping on to the paper strips. Levelling of the buffer was brought about by means of a connecting tube between the electrode compartments, which could be closed. The electrophoresis cabinets were finally placed in a refrigerator at 4° and a potential gradient of 10 volts/cm of paper applied at the electrodes, for eight hr.

Evaluation of the protein distribution. When the time of electrophoresis was complete the paper strips were taken out of the apparatus and heated in an oven at 110° for 30 min. After heating, the strips were stained to reveal the protein fractions by the method of JENCKS, JETTON and DURRUM (1954) using bromo-phenol blue, the dye being subsequently eluted with 0·01 N-sodium hydroxide in water. An elution method was preferred to densitometry of the strips as being more convenient and precise; in addition, the fractions did not have to form straight bands. The eluted dye from each of the fractions was made up to 10 ml with the dilute alkali, and the optical density read at 5900 Å. The figures for dye absorption were converted to the equivalent values of protein by means of a calibration curve, which was re-evaluated for each batch of extracts from different brains. Small volumes of the extract from grey matter, containing 10 to 500 μg of protein, were streaked on to a strip of paper similar to those used for electrophoresis. This was heated and stained together with the electrophoresis strips, and the dyed areas cut out and dye eluted. The figures for the optical density of the eluted dye, in 10 ml, were graphed as abscissae, (linear plot), against the known values for the protein adsorbing the dye (logarithmic plot). A shallow curve, and sometimes a straight line, connected dye absorption with protein content.

The electrophoretic separations were run in batches each day, until a total of at least thirty strips was obtained for each of the ten brains examined; five or more for each of the extracts from the different parts. Altogether 368 electrophoretic strips were used to provide the data shown in Table 1. The brains were those of patients who had died in hospital of causes unconnected directly with the

for detecting the presence of substances capable of exerting a lytic action on these tissues, and for their semi-quantitative estimation.

SUMMARY

1. A study has been made of the action of lysolecithin and Black Cottonmouth moccasin venom on whole-cell preparations (slices) of rat brain and liver and on rat quarter-diaphragms.

2. Both lysolecithin and venom cause a rapid and striking release of glutamic–oxaloacetic transaminase from each of these tissues.

3. Lysolecithin has also been shown to cause a somewhat smaller release of true cholinesterase from rat brain slices.

4. In confirmation of results reported by Quastel (1958) it has been shown that the respiration of rat brain slices is more sensitive to inhibition by the venom than is the respiration of either liver or diaphragm. O_2 uptake by brain slices is also more rapidly inhibited by lysolecithin than is the case with muscle or liver.

5. The partition of GOT activity between the various subcellular fractions derived from rat brain has also been described.

Acknowledgements—Our thanks are due to Dr. W. N. Aldridge and Mr. M. K. Johnston for kindly providing the brain fractions, to Mr. A. A. Cernik for his technical assistance, and to the Medical Research Council for providing a grant towards the expense of this work.

REFERENCES

Aldridge W. N. and Johnson M. K. (1959) *Biochem. J.* **73,** 270.
Birkmayer W. and Neumayer E. (1957) *Dtsch. Z. Nervenheilk.* **177,** 117.
Braganca B. M. and Quastel J. H. (1953) *Biochem. J.* **53,** 88.
Cabaud P., Leeper R. and Wroblewski F. (1956) *Amer. J. clin. Path.* **26,** 1101.
Cohen P. P. and Hekhuis G. L. (1941) *J. biol. Chem.* **140,** 711.
Dickens F. and Greville G. D. (1935) *Biochem. J.* **29,** 1468.
Edwards S. W. and Ball E. G. (1954) *J. biol. Chem.* **209,** 619.
Hanzon V. and Toschi G. (1959) *Exp. Cell Res.* **16,** 256.
Hird F. J. R. and Rowsell E. V. (1950) *Nature, Lond.* **166,** 517.
Hughes A. (1935) *Biochem. J.* **29,** 437.
Long C. and Penny I. F. (1957) *Biochem. J.* **65,** 382.
Marples E. A., Thompson R. H. S. and Webster G. R. (1959) *J. Neurochem.* **4,** 62.
Morrison L. R. and Zamecnik P. C. (1950) *Arch. Neurol. Psychiat., Chicago* **63,** 367.
Müller A. F. and Leuthardt F. (1950) *Helv. chim. acta* **33,** 268.
Nathan P. and Aprison M. H. (1955) *Fed. Proc.* **14,** 106.
Nygaard A. P. (1953) *J. biol. Chem.* **204,** 655.
Nygaard A. P., Dianzani M. U. and Bahr G. R. (1954) *Exp. Cell Res.* **6,** 453.
Nygaard A. P. and Sumner J. B. (1953) *J. biol. Chem.* **200,** 723.
Petrushka E., Quastel J. H. and Scholefield P. G. (1959) *Canad. J. Biochem. Physiol.* **37,** 975.
Quastel J. H. (1958). Proc. IVth. Int. Congr. Biochem. p. 90. (Vienna, Sep. 1958).
Rhodes D. N. and Lea C. H. (1957) *Biochem. J.* **65,** 526.
Sanders M., Akin B. A. and Soret M. G. (1954) *Acta neuroveg.* **8,** 362.
Saunders L. and Thomas I. L. (1958) *J. Chem. Soc.* 483.
Schneider W. C. (1956) Proc. III rd. Int. Congr. Biochem. p. 305. (Brussels, Aug. 1955).
Stern J. R., Eggleston L. V., Hems R. and Krebs H. A. (1949) *Biochem. J.* **44,** 410.
Tookey H. L. and Balls A. K. (1956) *J. biol. Chem.* **220,** 15.
Toschi G. (1959) *Exp. Cell Res.* **16,** 232.
Weil A. and Heilbrunn G. (1941) *Proc. Soc. exp. Biol., N.Y.* **48,** 233.
Weil A., Luhan A. J. and Balser B. H. (1935) *Trans. Amer. neurol. Ass.* **61,** 142.
Witter R. F., Morrison A. and Shepardson G. R. (1957) *Biochim. et biophys. acta* **26,** 120.
Wolfgram F. and Rose A. S. (1958) *J. Neuropath.* **17,** 399.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 145–149. Pergamon Press Ltd. Printed in Northern Ireland

THE PAPER ELECTROPHORETIC DISTRIBUTION OF SOLUBLE PROTEINS IN DIFFERENT REGIONS OF HUMAN BRAIN

D. M. ROBERTSON
M.R.C. Neuropsychiatric Research Unit, Whitchurch Hospital, Cardiff

(*Received* 31 *March* 1959)

A FEW investigators have published results in recent literature of their findings on the quantitative distribution of protein fractions from human brain tissue, separated by paper electrophoresis after extraction in aqueous media. Data known to the present writer include those of KAPS (1954) who made some studies, using paper electrophoresis, of protein changes in brain oedema. He found six fractions of soluble protein and reported some differences in the quantitative distribution of these in the neighbourhood of tumours. HOFMANN and SCHINKO (1956) investigated the protein distribution in white and grey matter by paper electrophoresis, but could find no differences. These authors gave evidence for the presence of lipid and carbonydrate attached to their fractions, and described some enzymes in the different fractions. KIYOTA (1956) extracted protein from the grey matter of normal and pathological human brains and separated this into six fractions by paper electrophoresis. He found that the electrophoretic pattern of protein from the grey matter of patients who had epilepsy, tumours or collagen disease showed greater amounts of 'albumin' than normal tissue. This was found to be true of immature brain as well. BOOIJ (1957) presented evidence that the electrophoretic 'pre-albumin' fraction encountered in work on the paper electrophoresis of proteins of the central nervous system, originates in the cerebrospinal fluid. According to this worker the 'pre-albumin' fraction is absent from extracts of white and grey matter. KARCHER, VAN SANDE and LOWENTHAL (1958) found differences in the quantitative distribution of protein in extracts from different parts of human brain, by the application of a small-scale electrophoretic technique on agar gel. These workers report the occurrence of two 'pre-albumin' fractions in some of their preparations.

The purpose of the present investigation was to find the amount of extractible protein obtainable from each of six different regions of human brain, by the use of a standardized extraction technique, and to find whether the quantitative distribution of the protein fractions separated by paper electrophoresis from these extracts showed differences.

METHODS

Extraction of protein. The method used for the extraction of the soluble proteins was essentially to make a homogenate of the tissue with the buffer used for the electrophoresis (1 g brain/ml buffer), to centrifuge the homogenate and use the supernatant fluid directly for paper electrophoresis. Extracts were made of frontal grey matter, white matter, cerebellum, pons, hypothalamus and medulla. As far as possible the pieces of tissue were taken from the same spots in the different brains. They were rinsed in distilled water, allowed to drain on hardened filter paper, homogenized and the homogenates centrifuged at 12,000 **g** for 20 min. About 0·2 ml of the supernatant fluid was acidified with 10%

trichloroacetic acid and the nitrogen content of the acid-insoluble material found by the micro-Kjeldahl method. The remainder of each extract was used for five or more paper electrophoretic separations of soluble protein, any material left over after four days being discarded. Freezing of the tissue before extraction, as recommended by HOFMANN and SCHINKO (1956) was not carried out as the degree of separation achieved was considered to be adequate. Freezing improves both the clarity of the electrophoretic pattern and the degree of extraction of the protein. Concentration of dilute extracts of the soluble proteins from brain tissue by dialysis against agar or dextran was not carried out, and undesirable changes in the protein pattern were brought about by this method. HAMMARSTON (1952) has reported that serum globulin is adsorbed on the walls of cellophane dialysis tubing during concentration of serum in dextran solution.

The electrophoretic separations were carried out in two perspex (acrylic resin) cabinets. Each was so constructed that eight paper strips, 5 cm by 30 cm, could be arranged in the same horizontal plane at the same height above the electrode compartments. The strips were individually stretched taut by shot-filled glass capsules in such a way that sag was negligible. The behaviour of chromatography paper as a supporting medium for the electrophoresis of the brain proteins was much improved by soaking it, for at least 12 hr, in the buffer before electrophoresis.

Paper strips, 5 cm in width and about 40 cm in length were cut from sheets of Whatman No. 2 chromatography paper. These strips were allowed to soak overnight or for at least 12 hr in the following buffer: 20·6 g of soluble barbitone (sodium diethyl barbituric acid) and 1·3 g of citric acid, in 2000 ml of distilled water. The components of the buffer dissolve rapidly if they are added separately. The pH of this buffer is 8·6 and the molarity, with respect to the sodium salt, 0·05 M. When the extracts of protein were ready for electrophoresis, the paper strips were taken out of the buffer and hung out of contact with one another in an enclosed space, to drain for about 20 min. After placing the strips in position in the apparatus, extract (0·1 ml) was applied on the edge of each of eight cover glasses to the eight strips in each cabinet, in a line about 5 cm from the negative end of the paper. The cover glasses were supported by leaning them against a rod running the width of the apparatus, and which was removed when the extracts had drained into the zone of application on the paper. A light spray of the cold buffer solution was applied to the paper from the positive end up to the point where the protein was applied, until the surface of the paper appeared wet. This helped to eliminate buffer flow from the ends of the paper towards the centre, thus preventing any disturbance of the position of the protein on the paper. When all the protein samples were applied, 500 ml of cold electrophoresis buffer were poured into each electrode compartment and the apparatus was closed with a tightly-fitting lid. Blotting paper on the underside of each lid prevented condensed moisture from dropping on to the paper strips. Levelling of the buffer was brought about by means of a connecting tube between the electrode compartments, which could be closed. The electrophoresis cabinets were finally placed in a refrigerator at 4° and a potential gradient of 10 volts/cm of paper applied at the electrodes, for eight hr.

Evaluation of the protein distribution. When the time of electrophoresis was complete the paper strips were taken out of the apparatus and heated in an oven at 110° for 30 min. After heating, the strips were stained to reveal the protein fractions by the method of JENCKS, JETTON and DURRUM (1954) using bromo-phenol blue, the dye being subsequently eluted with 0·01 N-sodium hydroxide in water. An elution method was preferred to densitometry of the strips as being more convenient and precise; in addition, the fractions did not have to form straight bands. The eluted dye from each of the fractions was made up to 10 ml with the dilute alkali, and the optical density read at 5900 Å. The figures for dye absorption were converted to the equivalent values of protein by means of a calibration curve, which was re-evaluated for each batch of extracts from different brains. Small volumes of the extract from grey matter, containing 10 to 500 μg of protein, were streaked on to a strip of paper similar to those used for electrophoresis. This was heated and stained together with the electrophoresis strips, and the dyed areas cut out and dye eluted. The figures for the optical density of the eluted dye, in 10 ml, were graphed as abscissae, (linear plot), against the known values for the protein adsorbing the dye (logarithmic plot). A shallow curve, and sometimes a straight line, connected dye absorption with protein content.

The electrophoretic separations were run in batches each day, until a total of at least thirty strips was obtained for each of the ten brains examined; five or more for each of the extracts from the different parts. Altogether 368 electrophoretic strips were used to provide the data shown in Table 1. The brains were those of patients who had died in hospital of causes unconnected directly with the

VOL. 5 1959/6

TABLE 1.—THE DISTRIBUTION OF SOLUBLE PROTEINS IN DIFFERENT PARTS OF THE HUMAN BRAIN. MEAN VALUES (± S.E.M.) FOR PROTEIN FACTORS SEPARATED BY PAPER ELECTROPHORESIS

Zone number from origin (origin = 0)	Analogous serum protein	Relative migration rate	Grey Matter (60 strips)			White Matter (64 strips)			Cerebellum (48 strips)			Pons (56 strips)			Hypothalamus (76 strips)			Medulla (64 strips)		
			Protein content (%)	S.E.M. for protein content (%)	protein content (μg)	protein content (%)	S.E.M. for protein content (%)	protein content (μg)	protein content (%)	S.E.M. for protein content (%)	protein content (μg)	protein content (%)	S.E.M. for protein content (%)	protein content (μg)	protein content (%)	S.E.M. for protein content (%)	protein content (μg)	protein content (%)	S.E.M. for protein content (%)	protein content (μg)
1	α-globulin	28	7·9	0·29	54	8·1	0·27	43	8·0	0·33	81	8·5	0·28	78	7·5	0·24	47	8·8	0·38	68
2	β-globulins	39	8·1	0·20	56	8·3	0·27	44	8·2	0·28	83	9·8	0·45	91	5·9	0·30	39	6·8	0·34	51
3		47	14·2	0·42	100	14·3	0·37	76	13·9	0·53	143	13·4	0·57	124	11·5	0·40	76	10·8	0·40	88
4		56	24·3	0·50	166	20·9	0·84	113	19·1	0·71	194	25·0	0·65	230	20·4	0·42	135	15·0	0·61	120
5	α-globulins	65	15·6	0·16	110	17·6	0·73	103	16·6	0·40	171	15·2	0·54	141	13·3	0·29	87	11·4	0·36	92
6		72	11·8	0·39	82	12·7	0·56	68	12·6	0·33	132	12·6	0·53	116	15·6	0·54	100	16·3	0·60	129
7		81	12·5	0·51	85	15·5	0·85	82	13·7	0·94	141	11·5	0·80	106	20·2	0·67	133	28·9	0·99	231
8	albumin and prealbumin	105	5·6	0·45	39	4·6	0·35	25	6·7	0·52	67	4·5	0·52	41	3·9	0·45	26	2·6	0·14	21

brain; their ages ranged from 27 to 81 years and half were female. As far as this could be ascertained none of the brains was treated later than 48 hr after the time of death of the patient. Three representative protein patterns are shown in Fig. 1.

RESULTS AND DISCUSSION

On application of the standardized method of extraction described, the amounts of protein taken up into unit volume of solution were: grey matter, 14·5 mg; white matter, 13·1 mg; cerebellum, 19·9 mg; pons, 9·1 mg; hypothalamus, 10·2 mg and medulla, 13·2 mg. It is evident that cerebellum is much richer in soluble protein than any of the other regions examined. Each stained fraction on the electrophoresis strips was numbered 1–8, omitting the origin, and the distance migrated from the point of application was expressed as a precentage of the distance travelled under the same conditions by the albumin of 0·08 ml of normal human serum. In Table 1, the first three columns give the numbers of the protein fractions, the analogous serum proteins and the relative migration rates, respectively. The remainder of the table shows the quantitative data for the different parts of the brain. The percentage distribution of the proteins on each strip was found from the calibration curve, and the mean values found for all strips made from each of the six regions of the brain. The standard error of the mean (S.D./$\sqrt{n}$) of the values for percentage protein distribution were also found. The values for the actual mean protein content of each fraction were worked out to the nearest μg.

It may be seen, from the values given for the percentage distribution of white and grey matter, that fraction 4 in grey matter is relatively greater than in white matter, and that fraction 7 appears to be greater in white matter. Since haemoglobin migrates to the same position as fraction 4 and is present in greater amount in extracts of grey matter than in white matter, the difference in the case of this fraction between the two regions of the brain may be ascribed solely to haemoglobin. An extract of the brain of a rat which had been perfused with saline through the carotid arteries, and an extract of the brain of a rat not so treated, were found to differ only in the amount of protein in fraction 4.

While the percentage concentration of fraction 7 in white matter is greater than in grey, the absolute values for the protein content show that this fraction is present in very nearly equivalent amount in both extracts, and that the difference lies in the lesser amounts of the other seven fractions in white matter. The percentage distributions of white matter and of cerebellum have very nearly the same values, but this conceals the fact that the extract of cerebellum contains almost twice the amount of each of the fractions of soluble protein. Again, although the percentage distribution of fraction 7 in cerebellum is 13·7 per cent compared with 20·2 per cent in the hypothalamus, the actual amount of protein is greater, being 141 μg compared with 133 μg in the hypothalamus.

Much variation in the amount of protein in fraction 7 ('albumin') was encountered from brain to brain, particularly in extracts from hypothalamus, cerebellum and medulla. Double 'pre-albumin' fractions occurred in most of the extracts studied. An example of an electrophoretic separation of soluble protein containing double pre-albumin fraction is shown in Fig. 2. No gross difference in the protein distributions of the eight fractions, separated by paper electrophoresis from extracts of the soluble proteins of the different areas of the brain, were found.

VOL. 5 1959/6

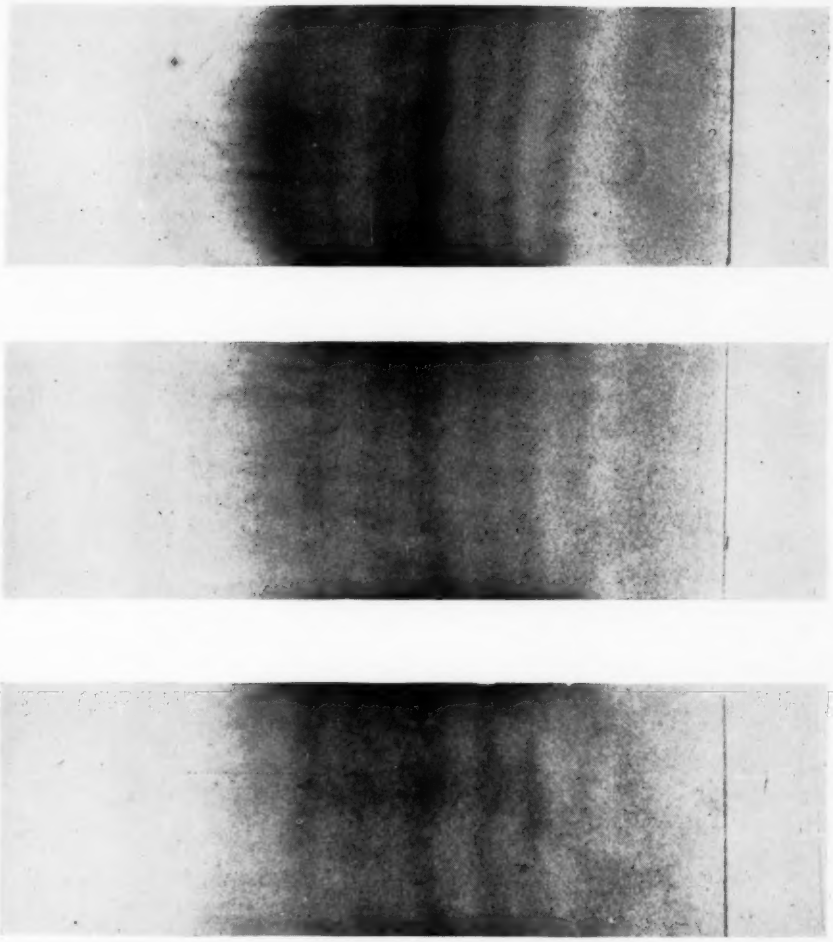


FIG. 1.—Representative patterns of soluble protein distribution by paper electrophoresis of (a) hypothalamus, (b) white matter and (c) grey matter from normal human brain.

FIG. 2.—An example of double pre-albumin formation in an electrophoretic separation of the soluble protein in an extract of normal human cerebellum.

VOL.
5
1959/6

Acknowledgements—The author thanks Dr. D. RICHTER for his advice, and also the post-mortem room staffs of Whitchurch and St. David's Hospitals, Cardiff, Wales. He is indebted to the Medical Research Council for a personal grant.

SUMMARY

1. A method for the paper electrophoretic separation of the soluble proteins of human brain is described. Good separation of the 'globulin' fractions of brain protein was achieved, using aqueous extracts of tissue.

2. Results obtained from the application of this method to the examination of six different regions of human brain, from normal patients, are presented and discussed.

REFERENCES

BOOIJ J, (1957) *Acta physiol. pharm. néerl.* **6,** 648.
HAMMARSTON G. (1952) *Scand. J. clin. Lab. Invest.* **4.**
HOFMANN G. and SCHINKO H. (1956) *Klin. Wschr.* **34,** 86.
JENCKS W. P., JETTON M. R. and DURRUM E. L. (1954) *Biochem. J.* **60,** 2.
KAPS G. (1954) *Arch. Neurol. Psychiat., Chicago* **192,** 115.
KARCHER D., VAN SANDE M. and LOWENTHAL A. (1958) Paper read at the IV International Congress of Biochemistry, Vienna, 1958. Proceedings published by Pergamon Press, London.
KIYOTA K. (1956) *Psychiat. et Neurol. jap.* **58,** 191.

Journal of Neurochemistry, 1960, Vol. 5, pp. 150–155. Pergamon Press Ltd. Printed in Northern Ireland

SODIUM, POTASSIUM, CHLORIDE AND WATER CONTENT OF SIX DISCRETE PARTS OF THE MAMMALIAN BRAIN*†

M. H. APRISON, ARLENE LUKENBILL and WILLIAM E. SEGAR
Institute of Psychiatric Research and Department of Pediatrics
Indiana University Medical Center, Indianapolis, Indiana

(*Received* 12 *June* 1959)

VOL.
5
1959/6

ELECTROLYTES, such as sodium, potassium and chloride, are known to affect the osmotic pressure, function and metabolism of nervous tissue. In order to understand these actions, it is necessary to determine the concentration of these ions in normal brain as well as in the discrete parts of the central nervous system (CNS). The pertinent CNS electrolyte data reported in the literature have been mainly on whole brain (MANERY, 1952; MANERY and HASTINGS, 1939; YANNET, 1939, 1940; YANNET and DARROW, 1938; LOWRY *et al.*, 1946; AMES and NESBETT, 1958) although there are a few reports on parts, such as cortex and cerebellum (MANERY and HASTINGS, 1939; EICHELBERGER and RICHTER, 1944; WOODBURY *et al.*, 1958). In addition, a single abstract has been published in which electrolyte data are reported on parts of the dog brain (TUPIKOVA and GERARD, 1937). Since neurochemical studies have already shown the wide range of metabolic activity associated with different brain areas, it appears that data on whole brain are of limited value. It is for this reason that the authors are presenting electrolyte data on six discrete major parts of the same brain. These specific parts were chosen because they represent various stages of phylogenetic development.

EXPERIMENTAL

Analytical techniques previously employed by this laboratory in muscle electrolyte studies were adapted to rabbit brain tissue (HOLLIDAY, 1955). Six adult, albino rabbits weighing approximately 4 kg were sacrificed by the intravenous injection of air. A craniotomy was performed, and the brain removed as quickly as possible. The specific brain areas were isolated and processed. Analyses were made of water, Na, K, and Cl content. Recovery studies were performed to check the accuracy of the analytical methods. These results were 103·0 ± 3·6% for Na, 97·0 ± 2·8% for K, and 99·2 ± 2·5% for Cl.

The brain samples were obtained from the following areas: *cortex* (grey), *caudate nucleus*, *thalamus*, *superior corpora quadrigemina*, *cerebellum*, and *medulla oblongata*. Right and left parts of the first four areas were combined. The cerebellar sample included the *vermis* and *lateral hemispheres*, but not the *paraflocculi*. When the brain was removed, it was separated from the spinal cord by making a transection just below the level of the *calamus scriptorius*. The medulla sample was the section between this level and the rostral end of the medulla cut to exclude the pons. A blood sample from each rabbit was obtained by cardiac puncture 24 hr prior to obtaining the brain parts. The serum was carefully separated and Na, K, and Cl determinations were made.

Calculations. Dry weight rather than fat free dry weight was chosen as the standard of reference for

* A preliminary report was presented at the Federation of American Societies for Experimental Biology, Atlantic City, New Jersey, April 16, 1959.

† This investigation was supported in part by the Riley Memorial Foundation and a grant (H-2883) from the National Institutes of Health, Public Health Service.

the calculation of total brain electrolyte and water content. This standard of reference was chosen because the lipids of brain form an integral part of functioning central nervous system tissue. Intracellular and extracellular water, Na, and K were calculated according to YANNET and DARROW (1940). These calculations are based on the assumption that Cl is confined to the extracellular space.

RESULTS AND DISCUSSION

The medulla, which is the oldest tissue from a phyletic point of view, was also the dryest; the cerebral cortex, which is the youngest, contained the most water (Table 1). The data for the other parts fell between these extremes. GRAVES and HIMWICH (1955) have reported on water content of four of the six parts studied in these

TABLE 1.—WATER (ml/100 gm) AND ELECTROLYTE CONTENT (m-equiv/100 gm) OF DRY BRAIN*

Part	H_2O	Na	K	Cl
Cortex	465 ± 21	34·8 ± 4·1	53·8 ± 2·2	24·2 ± 1·9
Caudate nucleus	423 ± 18	32·0 ± 2·1	47·1 ± 3·9	21·7 ± 2·3
Thalamus	303 ± 7	22·0 ± 2·2	36·0 ± 2·1	14·3 ± 0·5
S. c. quad.	341 ± 11	25·7 ± 2·3	39·3 ± 2·2	16·4 ± 1·5
Cerebellum	363 ± 9	27·2 ± 1·9	43·2 ± 1·8	16·7 ± 1·1
Medulla	261 ± 10	19·6 ± 1·5	31·9 ± 2·4	12·0 ± 0·8

* Data are expressed as the mean ±S.D. Brain parts from six albino adult rabbits were used in the analyses.

experiments. When the present data on water content of the cortex, caudate nucleus, superior corpora quadrigemina, and medulla oblongata were recalculated as percentage of wet weight, the results were in excellent agreement with those of GRAVES and HIMWICH. The water content for the thalamus and cerebellum did not seem to fit into the phylogenetic sequence. This was not unexpected since the thalamus is a mixture of old and new nuclei whereas the cerebellum develops phylogenetically with the cerebral cortex.

The total electrolyte content of the various brain parts paralleled the variation in water content (Table 1). Hence the total electrolyte concentration per litre of brain water remained relatively constant. When the data were calculated per 100 gm dry weight, the cerebral cortex contained the most Na, K, and Cl and the medulla the least. When the Na, K, and Cl data from Table 1 were each plotted against the total water content for a corresponding brain part, the resulting three functions were straight lines whose slopes are nearly the same. The slight differences in slope were due to the variation in percentage of extracellular and intracellular water in these different parts.

To understand better the metabolism of water and the electrolytes in organs and their subparts, knowledge of the composition of the extracellular and intracellular

fluids is necessary. It is possible to calculate the content of intracellular water and the intracellular concentration of K and Na in tissues such as muscle if the serum concentration of Cl and Na is known. Such calculations are based on two assumptions: (a) that Cl is an ion confined to the extracellular space, and (b) that serum ultrafiltrate can be used to measure the concentration of the extracellular electrolytes. However, when dealing with brain, two special problems must be considered before such calculations can be justified, First is the need to demonstrate that serum ultrafitrate is representative of the extracellular fluid of brain tissue. The second is the assumption that Cl is distributed largely or totally in extracellular fluid. This latter point is not agreed upon by all investigators (AMBERSON *et al.*, 1938). Recently, however, WOODBURY (1958) has shown that the Cl of the cerebral cortex of rat is in complete equilibrium with plasma. In experiments where isomolar glucose solutions were injected intraperitoneally into rats, the percentage change in brain Cl was equal to the percentage change in plasma Cl and the points fell along a line having a slope of unity. On the basis of these data Cl can be considered to be mainly extracellular in rat cerebral cortex. In addition, other evidence suggests that most, if not all, the Cl is extracellularly located in other CNS structures. Cerebrospinal fluid which is in intimate contact with the external surfaces of the brain is high in Cl concentration, as is the plasma of the deep blood vessels of the brain (MANERY, 1952). It has also been shown that components usually associated with being in the extracellular phase, such as connective tissue (MANERY *et al.*, 1938), dura mater (MANERY, 1952) and nerve sheath (WEBB and YOUNG, 1940) are high in Cl concentration. AMBERSON *et al.* (1938) and FENN *et al.* (1934) have shown that the Cl of vertebrate nerves appears to be extracellular.

The problem of deciding what fluid best represents extracellular fluid of the brain is still unresolved (MANERY, 1952; WOODBURY, 1958). According to our knowledge at present, the choice lies between plasma ultrafiltrate and spinal fluid. However, the difference between the two may not be important since, when equilibration has been established between blood and cerebrospinal fluid, their composition is similar.* Since serum samples are much more easily obtained than cerebrospinal fluid samples, and since the rabbits in the experiments described are in 'equilibration' as far as these two fluids are concerned, the former has been taken as representative of the brain extracellular fluid in the calculations of the data given in Tables 2 and 3.

Although extracellular water, intracellular water, and extracellular Na varied from high for cortex to low for the medulla, the amount of intracellular Na per unit of dry brain remained essentially constant for each of the six parts (Table 2). From these data, the volume of extracellular water, when calculated as percentage of wet weight, varied from 35·7 per cent for cortex to 27·4 per cent for medulla. The extracellular water content of the caudate nuclei was 34·4 per cent, whereas it constituted approximately 30 per cent of the total weight of the thalamus, the superior colliculus, and the cerebellum. The results obtained for cortex agree with those of other investigators (TOMAN and GOODMAN, 1947; HOLMES and TOWER, 1955; WOODBURY, 1958; LOWRY *et al.*, 1954). The ratio of intracellular to extracellular water increased from 1·28 in the cortex to 1·63 in the medulla. Although these data indicate that the extracellular space is much larger than that suggested by electron

* In the calculation of extracellular water from serum data, in addition to using the Gibbs-Donnan correction, the data are also corrected for the amount of solids present in serum.

VOL.
5
1959/6

microscopy data (DEMPSEY and LUSE, 1958), support for the present data is obtained from the cerebral impedence-conductance data of VAN HARREVELD and OCHS (1956, 1957) which suggest on the basis of a totally different group of experiments that the extracellular space of brain is of the order of 25 per cent. These authors have shown in some cleverly designed experiments that, following the death of the rabbit, the cerebral conductance decreases (impedence increases). The time interval following death during which the conductance decreases is of the order of 2·5–6 minutes. The

TABLE 2.—EXTRACELLULAR AND INTRACELLULAR DISTRIBUTION OF BRAIN Na AND WATER*

Part	H_2O_e†	H_2O_i†	Na_e‡	Na_i‡
Cortex	202	263	30·3	4·5
Caudate nucleus	180	243	27·0	5·0
Thalamus	119	184	17·7	4·3
S. c. quad.	136	205	20·4	5·3
Cerebellum	138	225	20·7	6·0
Medulla	99	162	14·8	4·6

* ml/100 gm dry brain
† m-equiv/100 gm dry brain
‡ n = 6
e = extracellular
i = intracellular

fall in conductance (rise in impedence) was explained by assuming that the extracellular water and electrolytes which are responsible for the passage of the current entered the cells. This assumption would explain the fact that the conductance fell after death, and could also explain the fact that the electron microscopist, who needs several minutes to obtain the tissue sample, finds very little space that can be identified as being extracellular. This phenomenon does not affect the present experiments since total Cl was measured and the assumption was made that it was all extracellular prior to death.

The sum of the intracellular Na and K concentrations (Table 3) was essentially the same for the various parts studied (about 219 m-equiv/l of intracellular water) whereas the ratio of the K concentration to Na concentration decreased from the anterior part of the brain (cortex = 13·4) to the posterior (medulla = 7·3). This change in ratio is due primarily to an increased concentration of Na in the intracellular fluid of the phylogenetically older portions of the brain. The increase in Na concentration is not large enough, however, to alter the sum of the Na and K concentrations in this fluid significantly.

Preliminary data suggest that there is a direct relationship between intracellular Na concentration and the total lipid expressed as percentage of wet weight. This relationship is currently under investigation.

Table 3.—Na and K concentration in 'intracellular' water*

Part	$[Na_i]$†	$[K_i]$†	$[Na_i] + [K_i]$‡	$[K_i]/[Na_i]$†
Cortex	16 ± 4	204 ± 8	220	13·4 ± 3·3
Caudate nucleus	19 ± 3	196 ± 11	215	10·5 ± 2·2
Thalamus	22 ± 4	196 ± 10	218	9·3 ± 2·0
S. c. quad.	24 ± 2	192 ± 9	216	8·2 ± 1·2
Cerebellum	27 ± 4	192 ± 5	219	7·4 ± 1·2
Medulla	28 ± 5	197 ± 1	225	7·3 ± 1·5

* All data are expressed as m-equiv/l; n = 6.
† Means with standard deviations.
‡ Sum of means.

VOL.
5
1959/6

SUMMARY

Na, K, Cl, and water content were determined in six discrete major parts of the adult rabbit brain, namely, cerebral cortex (grey), caudate nucleus, thalamus, superior corpora quadrigemina, cerebellum, and medulla oblongata. The total electrolyte content of the various parts paralleled the variation in water content. On a dry weight basis, the cerebral cortex contained the most Na, K, and Cl and the medulla oblongata the least. The data for the other parts fell between these extremes. Intracellular and extracellular water (ml/100 gm dry brain) and Na (m-equiv/100 gm dry brain) content were calculated on the basis of the two assumptions that Cl is extracellular and that plasma ultrafiltrate is representative of brain extracellular fluid. The justification of these assumptions was discussed. On the basis of these calculations, the sum of intracellular concentration of Na and K (m-equiv/l intracellular H_2O) remained constant, although the ratio of K to Na concentrations was approximately twice as great in the anterior as in posterior portions of the brain.

REFERENCES

Amberson W. R., Nash T. P., Mulder A. G. and Binns D. (1938) *Amer. J. Physiol.* **122,** 224.
Ames III A. and Nesbett F. B. (1958) *J. Neurochem.* **3,** 116.
Dempsey E. W. and Luse Sarah (1958) In *Biology of Neuroglia* (Edited by Windle W. F.) Chap. 7. Thomas, Springfield.
Eichelberger L. and Richter R. B. (1944) *J. biol. Chem.* **154,** 21.
Fenn W. O., Cobb D. M., Hegnauer A. H. and Marsh B. S. (1934) *Amer. J. Physiol.* **110,** 74.
Graves J. and Himwich H. E. (1955) *Amer. J. Physiol.* **180,** 205.
Holliday M. A. (1955) *J. clin. Invest.* **34,** 428.
Holmes J. M. and Tower D. B. (1955) In *Neurochemistry* (Edited by Elliott K. A. C., Page I. H. and Quastel J. H.) p. 285. Thomas, Springfield.
Lowry O. H., Hastings A. B., McCay C. M. and Brown A. N. (1946) *J. Geront.* **1,** 345.
Lowry O. H., Roberts N. R., Leiner K. Y., Wu M. L., Farr A. L. and Albers R. W. (1954) *J. biol. Chem.* **207,** 39.
Manery J. F. (1952) *The Biology of Mental Health and Disease,* Chap. 10. Hoeber, New York.
Manery J. F., Danielson I. S. and Hastings A. B. (1938) *J. biol. Chem.* **124,** 359.
Manery J. F. and Hastings A. B. (1939) *J. biol. Chem.* **127,** 657.

TOMAN J. E. P. and GOODMAN L. S. (1947) *Res. Publ. Ass. nerv. ment. Dis.* **26,** 141.
TUPIKOVA and GERARD (1937). *Amer. J. Physiol.* **119,** 414.
VAN HARREVELD A. and OCHS S. (1956) *Amer. J. Physiol.* **187,** 180.
VAN HARREVELD A. and OCHS S. (1957) *Amer. J. Physiol.* **189,** 159.
WEBB D. A. and YOUNG J. Z. (1940) *J. Physiol.* **98,** 299.
WOODBURY D. M. (1958) In *Biology of Neuroglia* (Edited by WINDLE W. F.) Discussion of Chap. 7. Thomas, Springfield.
WOODBURY D. M., ROLINS L. T., GARDNER M. D., HIRSCHI W. L., HOGAN J. R., RALLISON M. L., TANNER G. S. and BRODIE D. A. (1958) *Amer. J. Physiol.* **192,** 79.
YANNET H. (1939) *Arch. Neurol. Psychiat., Chicago* **42,** 237.
YANNET H. (1940) *Amer. J. Physiol.* **128,** 683.
YANNET H. and DARROW D. C. (1938) *J. clin. Invest.* **17,** 87.
YANNET H. and DARROW D. C. (1940) *J. biol. Chem.* **134,** 721.

OL.
5
9/60

Journal of Neurochemistry, 1960, Vol. 5, pp. 156–171. Pergamon Press Ltd. Printed in Northern Ireland

HISTOCHEMICAL INVESTIGATIONS ON SUCCINIC DEHYDROGENASE IN THE CENTRAL NERVOUS SYSTEM—IV

A HISTOCHEMICAL MAPPING OF THE CEREBRAL CORTEX OF THE GUINEA PIG

REINHARD L. FRIEDE
Bio-Acoustics Branch, Aerospace Medical Laboratory, Wright Air Development Center, Dayton, Ohio*

(*Received* 2 *October* 1959)

VOL.
5
1959/6

THE present article is a continuation of the histochemical mapping of succinic dehydrogenase activity (SDA) in the brain of the guinea pig (FRIEDE, 1959*a*); the cerebral cortex is described with particular reference to gradations of enzyme activity among cytoarchitectonic areas. The nomenclature and the spatial definition of the cortical areas of the guinea pig used in this article refer to ROSE's (1912) investigation of the cortex of certain small mammals. This article should be consulted for information on the cyto- and myeloarchitectonics of the cerebral cortex of the guinea pig, since the present paper will be limited to chemoarchitectonics. There is some disagreement, however, about ROSE's identification of areas (KRIEG, 1946) and about principles of architectonic mapping in general (LASHLEY and CLARK, 1946).

The histochemical architecture of the cerebral cortex is based mainly on gradations of the enzyme activity in the neuropil. The apparently independent behavior of enzyme activity in neuropil and in cell bodies stimulates the desire for further information on cytological localization of SDA. Two studies providing such information are now added to this investigation; one concerns the demonstration of SDA in isolated tissue elements and the other is concerned with correlations of glial cell density and enzyme activity in the neuropil.

MATERIAL AND METHODS

The description is based on five complete series of sections of the cerebral cortex of young adult guinea pigs. Three of these were cut frontally and two in an occipito-frontal direction. In addition, a great number of random sections was available. The specimens were orientated carefully so that the cortex was cut perpendicular to its surface. Since histochemical technique was the same as that used in the preceding studies of this series, the results in the cortex may be compared with those in other parts of the brain. Frozen sections, 60 μ thick, were cut from unfixed tissue and incubated in a medium containing tetrazolium, sodium succinate, and phosphate buffer of pH 7·8 for 2 hr at 38° (FRIEDE, 1958). Both tetrazolium derivatives were used: tetrazolpurpur (NEUMANN and KOCH, 1953) and Nitro BT (NACHLASS *et al.*, 1957). The results were identical with both derivatives, but if Nitro BT was used, the incubation time had to be shortened to obtain a staining intensity comparable to Tetrazolpurpur. The plates of this article demonstrate staining with Tetrazolpurpur except for Fig. 12 in which Nitro BT was used.

Densitometric measurements were used to establish quantitative differences in staining intensity. For these, an additional series of sections was cut carefully at 60 μ thickness and incubated in Nitro BT. Approximately equal volumes of tissue were incubated in each bottle and the bottles were

* Present address: Laboratory of Neuropathology, Neuropsychiatric Institute, University Hospital, Ann Arbor, Mich.

agitated during incubation. When mounting the sections with glycerine gelatin, identical weights were placed on the cover slips to avoid variable compression of the sections.

Densitometric measurements were made from 64 representative regions which are indicated in the text and in the plates. The measurements were taken on the screen of a Zeiss Ultraphot II with a Welch Densichron densitometer equipped with a blue CE-34 phototube. The densitometer was calibrated arbitrarily to show about 60 units in the putamen and 1·8 units on the open background. The intensity of SDA was expressed as percentage of that in the putamen, which was chosen as a reference because of its size and its rather uniform histochemical texture, and because it has been used frequently as a reference for quantitative determinations. However, contrary to the apparently homogenous staining of the putamen, densitometric measurements showed considerable gradations of enzymic activity. SDA was generally weaker in the lateral portion of the caudate nucleus than in its medial portion. Measurements, therefore, were made in a representative region in the centre of the head of the caudate nucleus.

In spite of the above precautions, the individual readings tended to be a little higher in some of the sections than in others. Slight variations in the thickness of the sections were probably responsible for this inconsistency, since the pattern of gradation within the sections was not changed. The arithmetic mean of an average of 93 readings was calculated for each region. It was felt that statistical calculation of a normal distribution was not permissible because of the slight variation of the material. The means obtained adequately reflected the gradations of staining intensity observed visually throughout the material. The readings are described as extinction values (E) in percentage of the putamen value; they are included in the text and are also shown in the plates which may be consulted for accurate localization. According to these readings, weak SDA was defined as below 30, mild SDA as 30–60, strong SDA as 60–90, and very strong SDA as above 90.

For the demonstration of succinic dehydrogenase in isolated tissue elements, carefully selected samples were taken from five adult guinea pigs and five adult rats from the following areas of the brain: cerebral cortex, superficial layers and deep layers, hippocampus, cerebral white matter, putamen, cerebellum, pons, medulla oblongata, and spinal cord. A tissue suspension containing single cells was obtained by gently shaking the immersed samples with glass beads with the technique of Chu (1954). The tissue disintegration was accomplished in the incubation fluid. The intimate contact with air during the shaking process did not impair the reaction as shown by comparison with anaerobically treated specimens. The same standard conditions of incubation were used as for tissue sections. The tissue suspensions were fixed in formalin, mildly counterstained by chromalum-gallocyanin, immersed in glycerin gelatin, and mounted; centrifugation of the suspensions preceded each of these steps of processing. Numerous isolated cells and small tissue fragments were found in the preparations. Other tissue suspensions incubated in Nitro BT were embedded in paraffin and studied in thin sections.

The technique employed for the investigation of correlations of glial cell density and enzyme activity in the neuropil will be described with the results, since this will help the interpretation of the experiment.

RESULTS

Isocortex

Some general rules of distribution of SDA prevail throughout the isocortex, whereas a variety of patterns is found in the allocortex. These rules are summarized preceding a description of the differences between the individual areas of the isocortex.

Mild SDA in the first or molecular layer is distributed diffusely in the neuropil; it is stronger in the superficial part of the layer than in the deeper part. No cell bodies are recognizable in the neuropil. The borders between the first or molecular layer and the second layer, though not sharp, are usually distinguished by an increase of SDA in the second layer. There is surprisingly little variation of SDA in the molecular layers of the individual areas (Table 1).

The second to fourth layers will be referred to in the following as the upper cellular layers, and the deeper fifth and sixth layers as the deep cellular layers. This gross subdivision of the cellular layers is supported by the conspicuous histochemical

differences among these portions. The histochemical designation of the fourth layer, however, is controversial, if compared with its cyctoarchetectonic designation in some areas.

The upper cellular layers form a zone of diffuse SDA in the neuropil in which the individual layers are not distinguishable. The intensity of SDA shows considerable variation among cytoarchitectonic areas; these differences are established mostly by gradations of SDA in the upper cellular layers. Within the neuropil, SDA is distributed homogenously and it cannot be localized in particular cell components. A few cell bodies with SDA stronger than that in the neuropil are found inconsistently scattered in the upper cellular layers. These may represent the hyperchromic cells which appear normally in animal cortex. The dense cell population of the upper cellular layers otherwise is barely recognizable in the neuropil. The cytoplasm of these cells contains SDA equal to or less than that in the neuropil since cell bodies may become outlined from the neuropil by absence of staining. The ability to observe cell bodies in the neuropil is not influenced by variations of the incubation period nor by variations in the thickness of the sections. Even regions normally containing very weak SDA in the neuropil of the upper cellular layers, such as the inferior temporal and insular cortex, do not show cell bodies; cell bodies would be recognized easily in these regions if SDA were present in them.

The deep cellular layers are distinguished sharply from the upper cellular layers by the predominance of SDA in the cell bodies and much weaker SDA in the neuropil. With the exception of the frontal cortex, the deep cellular layers continue without considerable changes in enzyme distribution throughout the areas. The fourth layer is sharply distinguishable from the upper part of the fifth layer. The SDA in the nerve cells of the fifth layer is usually stronger than in those of the sixth layer. The fifth layer sometimes is subdivided into three sublaminae; the middle sublamina stands out by strong SDA in its large pyramidal cells, whereas SDA in the nerve cells of the upper and lower sublamina approximately equals that of the intervening neuropil. The neuropil shows mild SDA throughout all three sublaminae. The sixth layer exhibits weak SDA in its neuropil and mild SDA in its nerve cells which are readily recognizable within the weaker SDA of the neuropil, whereas the nerve cells in the lower part of the fifth layer show about the same mild SDA but are barely discernible from the equally mild SDA of the neuropil. The boundaries between the cortex and white matter (E 10) are sharp in most areas of the isocortex, but are unsharp in the frontal areas, where cells are found scattered between the fibre radiations.

All the areas of the isocortex show gradual transitions into each other, even if the differences of intensity and distribution of SDA are as conspicuous as those between the pre- and postcentral cortex. The zones of transition of individual areas are usually not so narrow as to determine the boundaries indisputably as one would draw a line.

Most of the chemoarchitectonic differences between the cytoarchitectonic areas of the isocortex result from gradations of enzymic activity in the neuropil which, thus, behaves like an individual tissue unit. The development of the neuropil is relatively independent from the density of myelinated fibres in the grey matter, since there is no evident correlation between enzyme distribution and the myeloarchitectonics of the cortex.

VOL.
5
1959/6

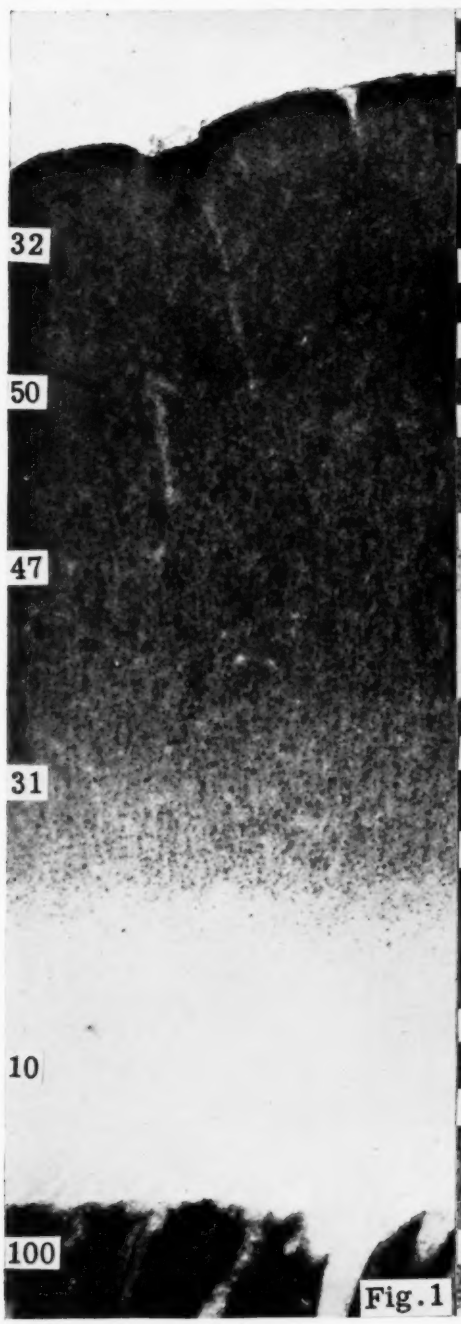


Fig. 1

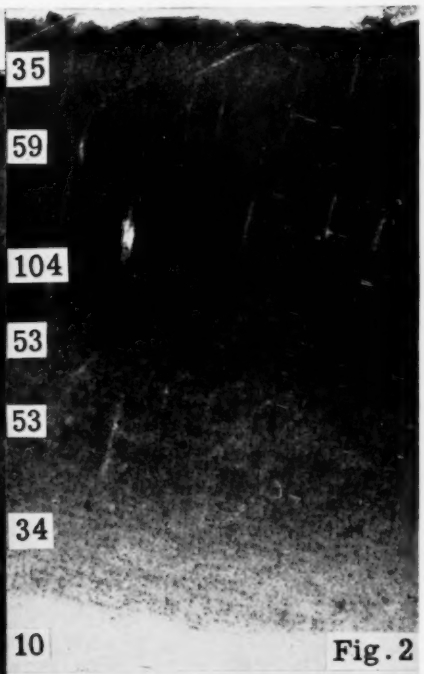


Fig. 2

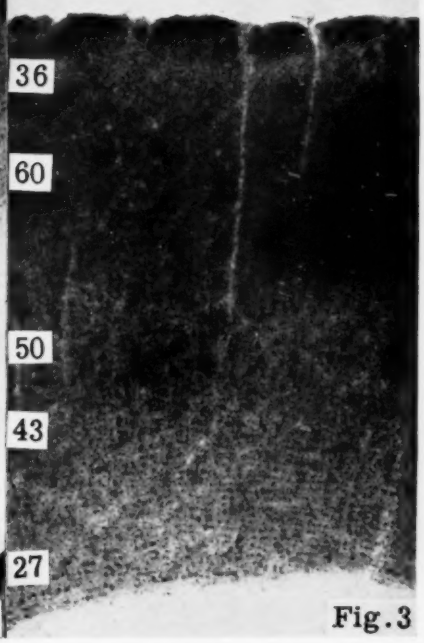
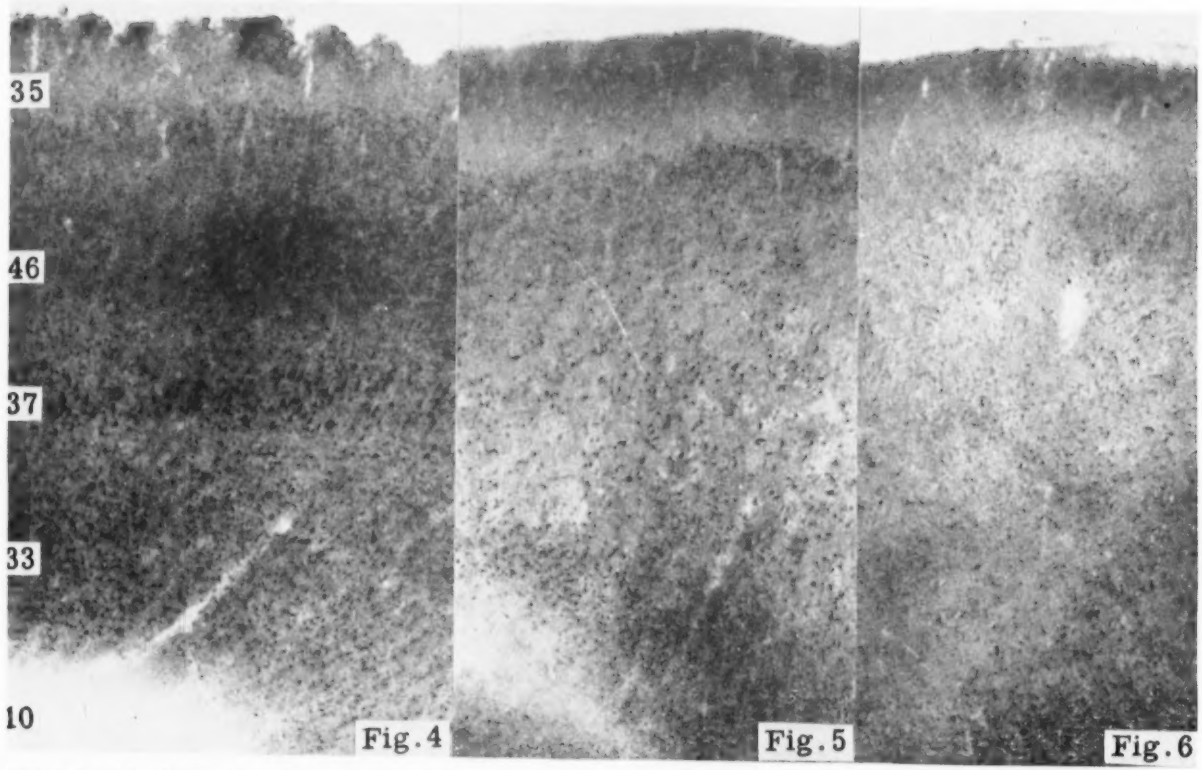


Fig. 3

FIG. 1.—Precentral region with white matter and a portion of the caudate nucleus. The numbers at the margin of the picture indicate the densitometer readings for this region.

FIG. 2.—Postcentral region, showing its typical segmentation of the third layer.

FIG. 3.—Transition of the postcentral region (right) into the parietal region (left) marked by decrease of enzymic activity in the third layer. The densitometer reading (60) refers to the parietal region in the left of the picture.



FIGS. 4, 5 and 6.—These three pictures are taken from the same section and indicate the gradual transition of the lower temporal region (Fig. 4) into the areas 35 (area perirhinalis; Fig. 5) and 28 (area entorhinalis; Fig. 6); enzymic activity is weak throughout these areas.

VOL.
5
1959/6

TABLE 1.—SUCCINIC DEHYDROGENASE ACTIVITY FROM DENSITOMETRIC MEASUREMENTS

	Areas	Molecular layer or first layer	Upper cellular layers	Deep cellular layers		Boundary of cortex and white matter	White matter	Fig.
				Fifth	Sixth			
Regio frontalis		32	50	47	31	Not sharp	10	1.
Area parorbitalis	8	50	66		35	Not sharp		10.
Regio postcentralis	1, 2, 3, 5	35	104	53	34	Sharp	10	2.
Auditory area	41		97			Sharp		
Regio parietalis	7	36	60	43–50–43 Sublaminae	27	Sharp	10	3.
Regio occipitalis	17, 18	35	93	60–52 Sublaminae	36	Sharp	10	
Inferior temporal and insular cortex		35	46	37	33	Sharp		4.
Regio cingularis	23		80 47 Gradual transition of the cellular layers			Sharp		
Area retrolimbica	29	71 Upper part 47 Lower part	75 45 Fourth layer 61 37			Not sharp		8.
Area parasubicularis	49		125 Merged second to fifth layers		51	Not sharp		7.
Area prepiriformis	51	87	43 ← Merged cellular layers →			Not sharp		10.
Tuber olfactorius	51	71	32 ← Merged cellular layers →			Transition into nucleus amygdalae		11.
Subiculum		114 23 White layer	75 ← Merged cellular layers →			Sharp	11	13.

SDA is expressed in per cent of the putamen; the data for the cornu ammonis, fascia dentata and olfactory bulb are included in the text.

Regio frontalis and precentralis (Fig. 1). The cortex of the precentral region is characterized by the mild SDA (E 50) in the thin upper cellular layers; a gradual increase of SDA from the frontal pole to the precentral areas can be established by photometric measurements.

The fifth layer (E 47) shows strong SDA in the large pyramidal cells which are widely scattered over the thick layer. The SDA in the intervening neuropil is only slightly weaker than that in the upper cellular layers. The pyramidal cells in the precentral cortex contain slightly stronger SDA than those in the frontopolar cortex and the formation to a fifth layer is more accentuated.

There is a gradual transition from the fifth to the sixth layer characterized by an offgrading of SDA (E 31) in both nerve cells and neuropil. At the frontal pole, however, there is little difference between the cells of the fifth and sixth layers. ROSE's identification of these areas with the areas 4 and 6 has been questioned for the rat (KRIEG, 1946).

Area 8 *or area parorbitalis* (Fig. 10, top). The *area parorbitalis* is discernible from the precentral and frontopolar cortex by stronger SDA in the upper cellular layers (E 66) and in the molecular layer (E 50), but there is a gradual transition between these areas which does not permit definition of a sharp boundary. The fifth layer shows a well defined lamina of large pyramidal cells with strong SDA.

Regio postcentralis (Fig. 2). The SDA in the upper cellular layers increases from the second layer (E 59) to the third layer which shows a segmentation into spherical zones of neuropil of very strong SDA (E 104) with intervening radii of mild SDA (E 53). This segmentation is characteristic for the postcentral region; it is more pronounced in its temporal extension than in its sagittal extension. That the zones of strong SDA are of spherical shape is concluded from the observation that their appearance is independent of the direction in which the area is cut. The segmentation of the third layer was found also in the rat, where it had already appeared in the cortex of 10-day-old rats.

The postcentral region (called area 1–3 and 5 by ROSE, 1912) shows histochemically no evident discrimination between the areas 1–3, but the area 5 (preparietal area) shows little or no segmentation of the neuropil in the third layer.

Regio parietalis, area 7 *or area parietalis* (Fig. 3). The *area parietalis* is distinguished from the postcentral region rather sharply by weaker SDA (E 60) in the upper cellular layers and the absence of an increase of SDA in the third layer.

Regio occipitalis, area 17, *or area striata and area* 18, *or area parastriata.* The distribution of SDA does not permit differentiation of areas 17 from 18. Both are characterized by very strong SDA in the neuropil of the upper cellular layers (E 93). Nerve cells with weak SDA are discernible frequently in the neuropil by absence of staining. There is neither a segmentation of the neuropil nor a particular increase of SDA in the third layer.

Regio temporalis. There seems a general agreement that ROSE was mistaken in the identification of the areas 20, 21 and 22, making no provision for the auditory cortex and attributing too great a region to the tempolar association areas. The histochemical findings in this region disagree considerably with ROSE's cytoarchitectonic map.

Area 41. The auditory area is well defined by very strong SDA (E 97) in the neuropil of the upper cellular layers. This very strong SDA shows a gradual transition

VOL. 5 1959/6

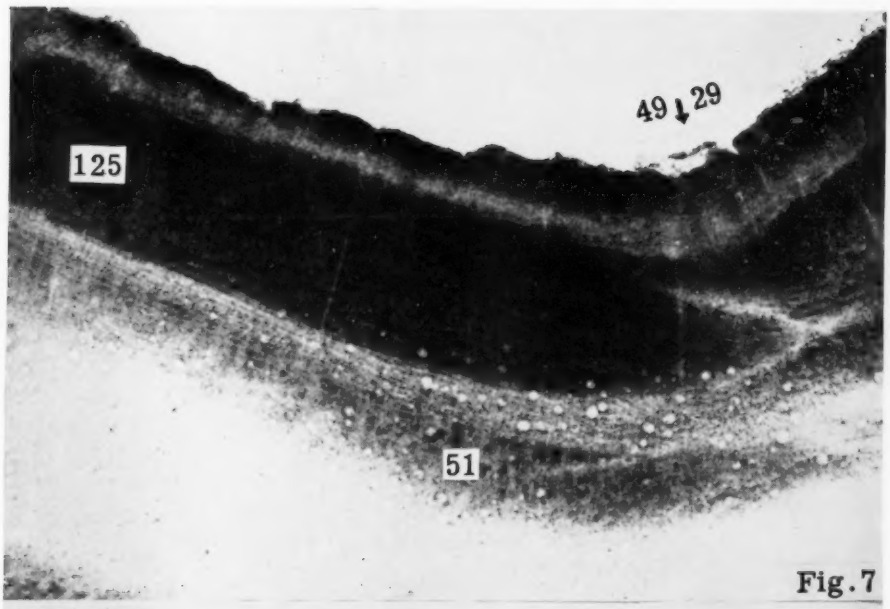


Fig. 7

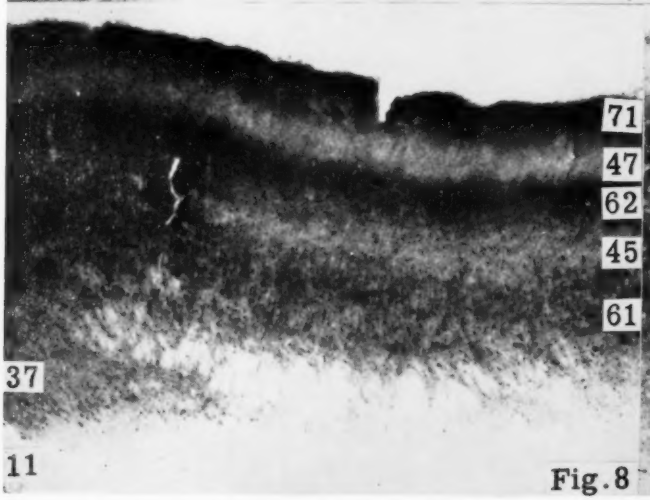


Fig. 8

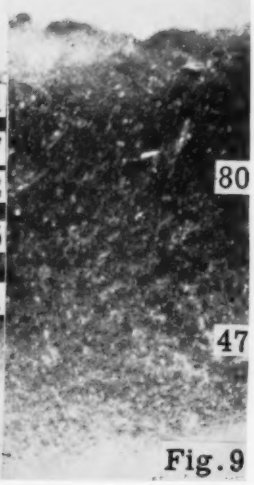
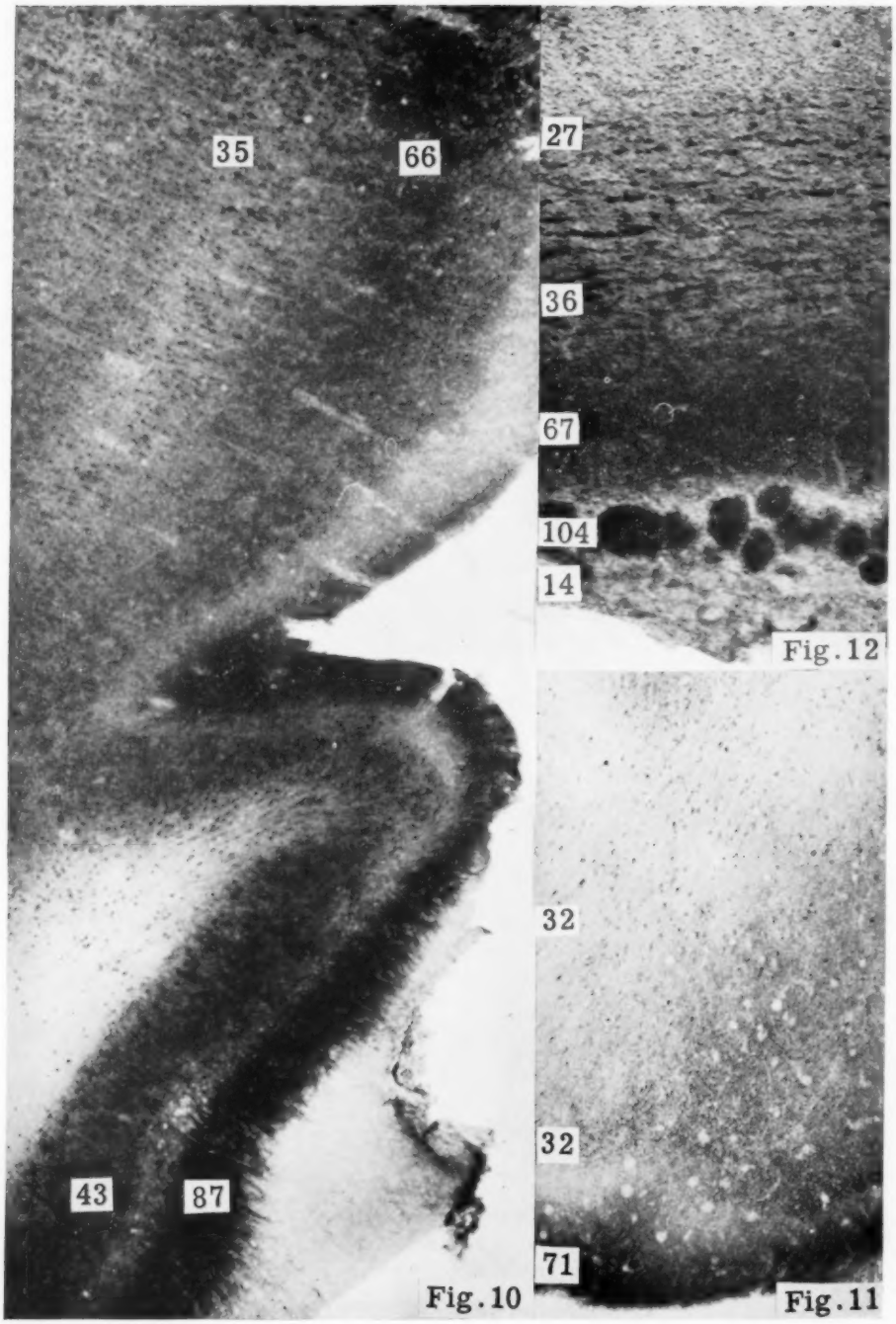


Fig. 9

FIG. 7.—Area 49 (area posthippocampica) and its sharp boundary at the area 29 (area retrolimbica) at the right of the picture.

FIG. 8.—Area 29 (area retrolimbica) with its typical trilaminate pattern.

FIG. 9.—Absence of a laminate distribution of enzymic activity in the area 23 (area limbica posterior).



FIGS. 10 and 11.—Distribution of enzymic activity in the area 51 (area prepyriformis). Fig. 11 demonstrates the particularly low enzymic activity in the cellular layer of the tuber olfactorius. The upper part of Fig. 10 shows the area 8.

FIG. 12.—Distribution of enzymic activity in the olfactory bulb.

VOL.
5
1959/6

into the postcentral region, but a differentiation is possible by the complete absence of the segmentation of the neuropil in the auditory area. Between the auditory area and the occipital areas, there is a narrow zone of weaker SDA connecting the parietal cortex with the inferior temporal cortex.

Inferior temporal and insular areas (Fig. 4). These areas, designated areas 20 and 13–16 by ROSE, are not histochemically distinguishable from each other, but are demarcated clearly from the auditory area by the very mild SDA in them. They also merge with the adjacent areas 36 and 35 and parietally with the area 7. Since there is a very gradual increase of SDA from the area 35 to the parietal region, all these areas form a temporo-parietal band with little or no boundaries between individual areas. It is a characteristic of the inferior temporal and insular cortex for the SDA in the upper cellular layers (E 46) to be not much stronger than that in the molecular layer (E 35) and in the deep cellular layers (E 37). The fifth layer is the most conspicuous of the cortex by its lamina of large pyramidal cells with strong SDA.

Regio cingularis. The areas of this region are defined by absence or very poor development of a laminar architectonics. The areas 23 and 24 (Fig. 9), or *areas limbica anterior* and *posterior*, are not discernible histochemically. The molecular layer exhibits weak SDA throughout. The cellular layers form a band of neuropil with strong SDA in the upper part (E 80) grading off (E 47) in the deeper part of the cortex without demarcation of individual layers. Cell bodies may be recognized in the deeper part of the cortex, particularly in the area 24. Cell bodies are discernible more readily in the area 32, or *area prelimbica*, where they show slightly stronger SDA than the neuropil. There are, however, no differences of SDA between the cells of the fifth and sixth layers. The area 25, or *area pregenualis*, is continuous with the area 24 and discernible only by the thickness of its layers. The very thick first layer with strong SDA in its superficial part makes the cortex appear to be bilaminate.

Allocortex

The distribution of SDA in the allocortex shows a considerable variability of patterns among the individual areas, most of which show much stronger SDA in the molecular layer than the isocortex.

Regio retrosplenalis, area 29 *or area retrolimbica* (Fig. 8). The *area retrolimbica* exhibts a typical pattern of three bands of strong SDA separated by zones of weaker SDA. The thick first layer shows strong SDA in its upper part (E 71) decreasing to mild SDA in its deeper part (E 47). The merged second and third layers show strong SDA in the neuropil (E 75) with almost no cell bodies discernible. In the fourth layer, SDA decreases (E 45) and some cell bodies become visible. The fifth layer is subdivided showing stronger SDA in the neuropil of its upper part (E 61) but weaker SDA in the neuropil of its deeper part. The SDA of the neuropil decreases in the sixth layer (E 37) where cell bodies with mild SDA are found.

The area 29 shows a gradual transition into the area 18 (at the cytoarchitectonic part C of the area 29) but there is an almost linear separation of the area 29 from both area 49 (Fig. 7) and the *subiculum* by a narrow zone of weak SDA; such a spatial separation of areas is unique in the cerebral cortex.

Regio hippocampica, areas 27 *or presubicularis and* 49 *or parasubicularis* (Fig. 7). SDA is definitely stronger in the area 49 than in the area 27, but its distribution is similar in both areas.

The thick molecular layer shows an accentuated gradation, from strong SDA in its superficial part to weak SDA in its deep part. The second to fifth layers are merged forming a band of very strong SDA (E 125, in area 49) in the neuropil. The upper part of this band is homologuous with the second and third layers and exhibits small nerve cells which are barely discernible from the neuropil by slightly stronger SDA. The deep part of this band is homologuous with the fifth layer and exhibits larger nerve cells readily discernible from the neuropil by stronger SDA. These merged layers are separated sharply from the adjacent first and sixth layers and also from neighbouring cortical areas. The sixth layer (E 51, in area 49) shows mild SDA in its neuropil and stronger SDA in its cell bodies.

Area 35 or area perirhinalis and area 36 or area ectorhinalis (Fig. 5). The histochemical differentiation of the *ectorhinal* and *perirhinal areas* from adjacent *inferior temporal areas* is poor. Differences among these areas are established by minor variation of the thickness of laminae or by the arrangement of cells in them, as weak SDA continues throughout all these areas. The pyramidal cells of the fifth layer are more widely spaced in the area 36 than in the *inferior temporal areas* and show slightly less SDA. The molecular layer of the area 35 is very thick and exhibits weak SDA. The deep cellular layers are narrow and the fifth and sixth layers are barely discernible.

Mild to weak SDA continues from the area 35 throughout the adjacent *area 28 or area entorhinalis* (Fig. 6). This area is clearly discernible, however, by the segmentation of the second layer into cell islands with weak to mild SDA. In the deeper part of the cortex, cells gradually scatter and the third to fifth layers are not discernible. The sixth layer is thick and contains scattered star-shaped cells with mild SDA in a neuropil of weak SDA. In spite of the striking differences in enzyme distribution, the transitions between area 28 and area 51 are very gradual.

Regio olfactoria, area 51 or area prepyriformis (Figs. 10 and 11). A typical bilaminate distribution of SDA characterizes the area 51 in which the very thick molecular layer shows the strongest SDA of all the layers (E 87). The cellular layers form a sharply delineated band of mild SDA (E 43) in the neuropil with nerve cells containing slightly stronger SDA. There are several cytoarchitectonic subdivisions of the area 51, but only the *tuber olfactorius* (subarea d) is demarcated clearly by weaker SDA in both molecular layer (E 71) and cellular layers (E 32). The deep part of the cortex shows large nerve cells with a considerable variability of their SDA content; these cells blend with the *nucleus amygdalae*.

Subiculum (Fig. 13). The narrow molecular layer shows very strong SDA (E 114); it is separated from the cellular layers by a thick layer of white matter with weak SDA (E 23). The large pyramidal cells of the subiculum exhibit strong SDA. The neuropil between these cells contains mild to strong SDA (E 75) with some variation among parts of the *subiculum*. The boundaries between the *subiculum* and the areas 29 and 27 are very sharp as has been described above.

Cornu ammonis (Fig. 13). VOGT's subdivision of the *cornu ammonis* into the parts H_1, H_2 and H_3 is supported by histochemical differences among these parts.

Part H_1: The white matter of the *alveus* lacks SDA (E 11). The *stratum oriens* shows mild SDA (E 55) in its neuropil. The *lamina pyramidalis* stands out by the strong SDA (E 90) of its cell bodies. SDA extends from the cell body into the apical dendrites for a distance not exceeding the thickness of the *lamina pyramidalis*. The

VOL. 5 1959/6

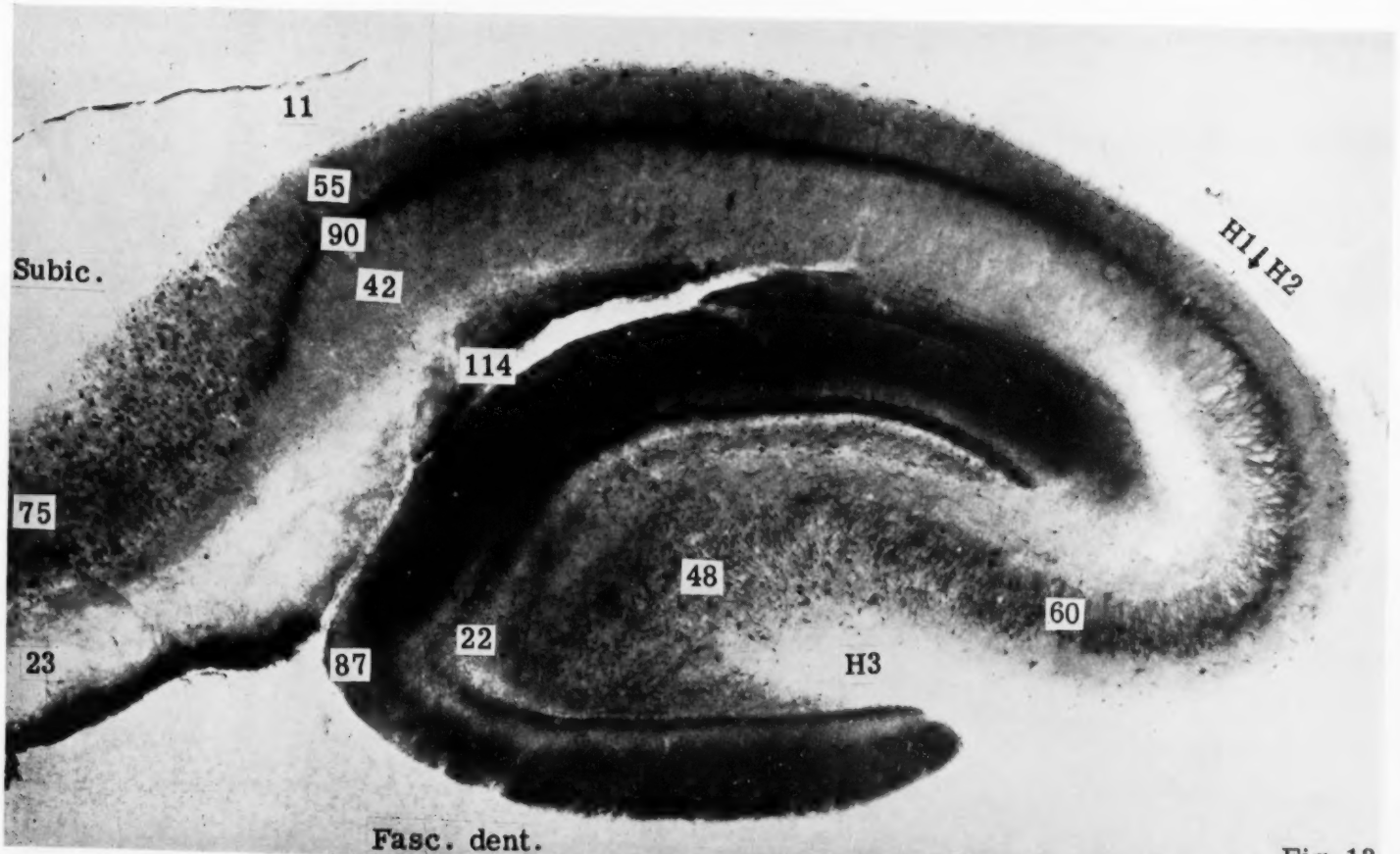


Fig. 13

FIG. 13.—Distribution of enzymic activity in the subiculum (Subic.), ammonshorn (H1, H2 and H3) and the fascia dentata (Fasc. dent.).

VOL.
5
1959/6

SDA of the neuropil is slightly weaker in the immediate proximity of the pyramidal cells. The mild SDA of the *stratum radiatum* (E 42) increases in the *lamina lacunaris* (E 87) and is very strong (E 114) in the *lamina molecularis*.

Part H_2 differs from H_1 in the distribution of SDA in the apical dendrites of the pyramidal cells. Many of these dendrites are discernible by strong SDA extending into them over a distance exceeding twice the thickness of the lamina pyramidalis. The neuropil between these dendrites shows weaker SDA than the rest of the *stratum radiatum*; the dendrites, therefore, are sharply demarcated from the faintly stained neuropil.

Part H_3: The particular staining of the apical dendrites disappears in part H_3 which is characterized by a scattering of the pyramidal cells and by an increase of SDA in the neuropil between these cells (E 60). No molecular layer of very strong SDA is found for H_3.

Fascia dentata (Fig. 13). The molecular layer of the *fascia dentata* is divided into a thick upper part with strong SDA (E 87) and a thin deep part with mild SDA in the neuropil. The dense *lamina pyramidalis* shows strong SDA in the cells similar to that in the *lamina pyramidalis* of H_1. The *lamina pyramidalis* is separated from the *lamina multiformis* by a narrow zone of weak SDA (E 22). Mild SDA is found in the neuropil of the *lamina multiformis* (E 48) containing scattered cell bodies with stronger SDA.

Olfactory bulb (Fig. 12). In the strata of the olfactory bulb SDA prevails in the neuropil and is weak in nerve cells. The terminology of the strata of the olfactory bulb refers to the atlas of WINKLER and POTTER (1911).

The *lamina fibrosum nervi olfactorii* or lamina I shows weak SDA (E 14). The *lamina glomerulosa* or lamina II exhibits very strong SDA (E 104) distributed diffusely in the *glomeruli olfactorii* (ORTMANN, 1957). The tissue between the glomeruli contains weak SDA but appears granulated, since a few of its cells show mild to strong SDA. The cell masses between the glomeruli are not discernible from the *lamina granulosa externa* or lamina III. The *lamina gelatinosa* or lamina IV shows strong SDA (E 67) diffusely distributed in the neuropil. The SDA is strongest in the centre part of the lamina decreasing in the superficial and deep parts. The large mitral cells of the *lamina cellularum* or lamina V show mild or weak SDA and are barely discernible from the adjacent laminae contrary to their outstanding appearance by conventional staining techniques. The *lamina granulosa interna* or lamina VI shows a considerable decrease of SDA (E 36) in the neuropil. The SDA of the VIth layer decreases towards the deep fibre layers (E 27) (*lamina fibrorum tractus olfactorii* or lamina VII and *lamina fibrorum medullae lobi piriformis* or lamina VIII). The ependyma of the *ventriculus pyriformis* exhibits strong SDA, as is typical for ependyma elsewhere in the ventricles.

Demonstration of succinic dehydrogenase in isolated tissue elements

Large neurons were isolated most frequently from the *medulla oblongata*. SDA was strong in the perinuclear part of the cytoplasm, it decreased towards the cell wall, and showed a further reduction in the dendrites. No SDA was found in the nucleus. Similar distribution of SDA was present in large neurons from other regions of the brain, such as in cerebellar Purkinje cells or in cerebral pyramidal cells. A mild or weak SDA, however, was frequently found in small neurons from many

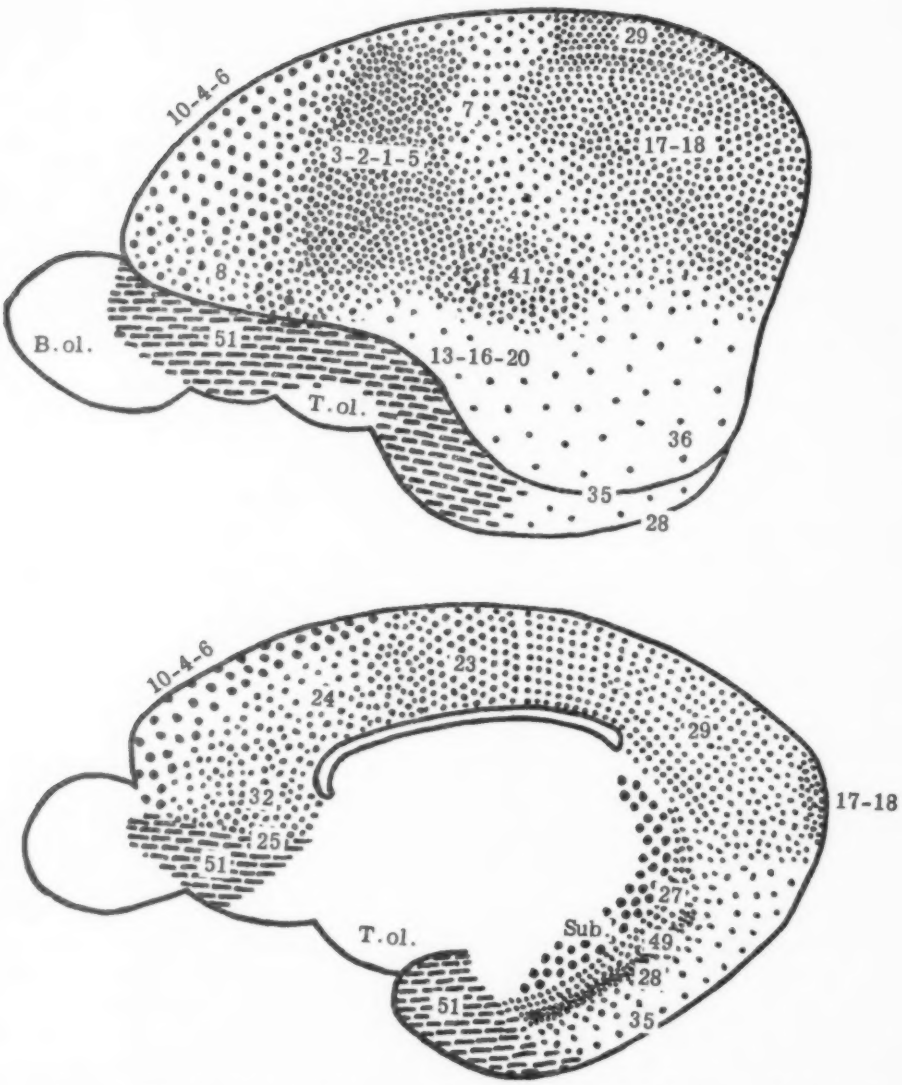


FIG. 14.—Histochemical map of the cerebral cortex of the guinea pig, indicating the distribution of succinic dehydrogenase activity. The density of dots reflects gradations of enzymic activity. No attempt is made to indicate linear boundaries between areas, since there are very gradual transitions between the areas. The numbers indicate the correlation to the cytoarchitectonic areas. The symbols used for the area 51 indicate that enzymic activity prevails in the molecular layer.

parts of the brain. Small neurons with weak or absent SDA were prevalent in samples from the upper cerebral layers and the putamen.

Most of the dendrite branchings remained included in fragments of neuropil. Isolated dendrites or long dendrites still connected to the cell body were less frequent and usually exhibited mild SDA.

VOL. 5 1959/6

Neuropil appeared as irregularly shaped tissue fragments showing about the same intensity of SDA as was found in tissue sections from the same area. Attempts to isolate tissue structures composing the neuropil failed. Large dendrites with SDA slightly stronger than in the neuropil were sometimes identified within the neuropil.

The tissue componenets of the cerebellar granular layer provided a better insight into the cellular distribution of SDA. The glomerula cerebellaria were isolated as globular corpuscles of about equal size and exhibited strong, uniformly distributed SDA. The cerebellar granular cells, on the other hand, showed very little SDA and only a few formazan granules were found in the cytoplasm or in the short dendrites. This inverse distribution of SDA in granular cells and in glomerula cerebellaria was established in both isolated cells and small tissue fragments. If a tissue fragment was depleted of glomerula cerebellaria, there was an almost complete absence of SDA; if it contained glomerula, they stood out individually by their strong SDA. The SDA in the bodies of granular cells, therefore, was definitely weaker than that in the synaptic glomeruli which contain the peripheral cell branchings. The recognition of this distribution gradient within the cell was facilitated by the specific structure of the glomerula cerebellaria; a similar distribution gradient is very likely for other regions of the brain such as the upper cortical layers or the putamen.

Glial cells isolated with all their processes were sparse, but isolated glial nuclei, nuclei with some cytoplasm attached, and small tissue fragments containing glial cells were abundant. Glial tissue from white matter lacked SDA. Glia from grey matter exhibited exceedingly weak, or no, SDA; only a few formazan granules were found in their cytoplasm.

Axons usually lacked SDA, but axons with a weak reaction were observed occasionally. SDA was never observed in myelin.

Capillary vessels and larger vascular arborizations were easily isolated. Exceedingly weak, or no, SDA was found in capillaries from white matter, which exhibited only a few scattered formazan granules in their walls. Stronger SDA was found in capillaries from grey matter, particularly the cerebellar or cerebral cortex. Such vessels were outlined by mild SDA in their walls, being stronger around the endothelial nuclei. Arterioles of both grey and white matter exhibited moderate to strong SDA in their smooth muscle cells, sharply outlining the muscular architecture of their walls.

Correlations of glial cell density and enzyme activity in the neuropil

The absence of enzyme activity in isolated glial cells seems to indicate little contribution of glial cells to the enzyme activity of the neuropil. The following findings, however, reveal a contradictory point of view.

Photomicrographs were taken from the magnocellular part of the reticular formation of the *medulla oblongata* where the neuropil shows a reticular arrangement between perforating fibre bundles. The sections were removed from the slide, dehydrated, and all the formazan was removed with alcohol–ether at 38° in 1–3 days. The sections were restained by chromalum–gallocyanin and photomicrographs were again taken. With nerve cells as landmarks, exactly identical fields were established for both SDA and gallocyanin stain. This technique was originally developed to investigate correlations between chromatin and SDA in nerve cells during axonal reaction (FRIEDE, 1959*c*). The areas with neuropil as defined by content of SDA

were outlined and transferred to the photographs demonstrating cell stain; these areas of neuropil did not include any pericarya of nerve cells. Areas with fibre bundles lacking SDA were transferred in the same way. The size of these areas was measured by a polar planimeter and the content of glial cells in them was counted. On the basis of more than 5000 counts, neuropil with SDA was found to contain almost exactly twice as many glial cells as were present in fibre bundles without neuropil.

DISCUSSION

The relationship of SDA and vascularization was discussed in the preceding article on the distribution of SDA in the guinea pig's midbrain. The cerebral cortex was included in this discussion as a preview of some of the results of the present investigation. The present discussion emphasizes the comparison of microchemical and histochemical data and the relationship of SDA to cell statistics. It is hoped that the latter part will elucidate the problem of enzyme localization in the neuropil.

The gradations of the formazan staining among various parts of the brain compare favorably with biochemical determinations of SDA in the dog's brain (BURGEN and CHIPMAN, 1951). The present investigation allows comparison of histochemical findings with quantitative microchemical data on enzymes in layers of the cerebral cortex. Since there are no such data available on succinic dehydrogenase, it seems advisable to compare other enzymes closely linked to it, such as fumarase or malic dehydrogenase. The enzymes of the glycolytic and the citric acid cycle bear a surprisingly constant relationship to one another throughout the depth of the cerebral cortex (ROBINS *et al.*, 1956).

The data of LOWRY *et al.* (1954) on the activity of fumarase in the layers of the *cornu ammonis* parallel the gradation of SDA found in the present investigation. The distribution of fumarase activity in the layers of the precentral and occipital cortex (ROBINS *et al.*, 1956) are also reflected by the distribution of SDA. There was a continuous decrease of fumarase activity from the second to the sixth layer in the precentral cortex, but in the visual cortex fumarase activity was strong from the second to the fifth layer and decreased sharply in the sixth layer. The strong fumarase activity of the fifth layer may be explained by the concentration of enzymic activity in the large pyramidal cells which is not adequately reflected in photometric readings.

Microchemical and histochemical findings also agree during development of the newborn rat's cerebral cortex; the changes of SDA as reported in the first article of this series (FRIEDE, 1959*a*) are paralleled by changes of malic dehydrogenase activity (KUHLMANN and LOWRY, 1956). The author's discrimination of three histochemically discernible primitive layers, A, B and C, in the newborn rat's cortex and the later disappearance of this primitive pattern is surprisingly well reflected in KUHLMANN and LOWRY's Fig. 4 which illustrates changes in malic dehydrogenase.

Since there is so close a correlation between the staining reaction for SDA and quantitative histochemical data on other enzymes of the Krebs cycle, the mapping of SDA seems a fairly reliable source of information on the activity of the citric acid cycle in areas of the guinea pig's brain.

NISSL (1898, 1903) has drawn attention to the fact that greater spacing of neurons is found in more highly developed than in primitive brains. Spacing of neurons may

VOL.
5
1959/6

be regarded as a parameter for the development of the neuropil. Quantitative determination of spacing of neurons was attempted first by v. ECONOMO (1926, grey cell coefficient) and later by v. BONIN (1950), SHARIFF (1953), HAUG (1953, 1956, 1958) and REBHAN (1956). The grey cell coefficient is a factor indicating the volume of nerve cell bodies in relation to the total volume of grey matter; it seems to rise with phylogenetic development, but the limiting factor may be more the size of the brain (TOWER, 1954) then its degree of development (HAUG, 1953).

A few investigations indicate a relationship of spacing of neurons to functional activity. Spacing of neurons increases during normal development and seems to be correlated with an increase of oxygen consumption (FLEXNER and FLEXNER, 1946). Reduced spacing (denser crowding of neurons than is normally found) has been described for a variety of conditions such as the homolateral visual cortex of animals with experimentally produced ankyloblepharon (BERGER, 1921), the human right motor region in comparison to the left (BECK, 1950), the cortex of experimentally hypothyroidic rats (EAYRS and TAYLOR, 1951, EARYS, 1953), idiocy (HAMMARBERG, 1895), the *nuclei acustici* of deaf subjects (SCHENK, 1947), or the aging of the *dentate nucleus* (HOEPKER, 1951). Quantitative foundation seems desirable for many of these observations.

In cortices, a part or all of the neuropil may be arranged in a molecular layer; these layers maintain a constant relationship to the cellular layers through cortical convolutions (BOK, 1929, FRIEDE, 1955*a*); more so as the volume of the cerebellar molecular layer seems to control the spacing of the Purkinje cells (FRIEDE, 1955*b*, BRAITENBERG and ATWOOD, 1958).

The amount of dendrite branching probably accounts for a major part of the spacing of neurons (CAJAL, 1906), but there is also some indication for a quantitative participation of glial tissue in spacing of neurons. The number of glial cells per nerve cell (glial index) increases during phylogenetic development (FRIEDE, 1954), but the limiting factor may be more the size of the brain (HAWKINS and OLSZEWSKI, 1957) than its degree of development, as has been indicated above for the grey cell coefficient. There is very little information on the relation between functional changes and glial index. The normal development of the brain seems to be accompanied by a considerable increase of the relative amount of glial tissue (MUEHLMANN, 1936, HAUG, 1958) and there seems also to be a continuous increase of glial tissue during the normal aging of the horse (BRAUN, 1951) and probably also of man (FERRERO, 1947).

There have been numerous attempts to define the neuropil (APATHY, 1897) as a specific tissue unit discernible from the cellular constituents of grey matter, such as 'koernige Grundsubstanz' (KOELLIKER, 1850); 'Nervennetz' (GERLACH, 1872); 'Neurenzytium or Grundnetz' (HELD, 1927); 'nervoeses Grau' (NISSL, 1898, 1903); and 'blasses Syncytium' (BAUER, 1938, 1943, 1953). The term 'ground substance' has been used frequently as a substitute for neuropil ('The ground substance appears to consist mainly of fine dendrite branchings,' DIXON, 1954). Confusingly, the term 'ground substance' has also been used for a truly intercellular substance of polysaccharide nature (HESS, 1953, 1955; OZZELLO, LENDING and SPEER, 1958). It is felt that any of these specific claims for the nature or constituents of the neuropil are beyond the resolving power of the techniques employed. For example, periodic acid–Schiff reactive material would appear to be extracellular if it accumulated

exclusively in processes of glial cells; this type of distribution, however, has been shown for the giant glial cells of the leech (FRIEDE, 1955*c*).

In summarizing these findings, it would seem that both dendrites and glial processes participate in the formation of the neuropil, the glial tissue probably to a smaller extent than the dendrites. A first quantitative estimate attributes 60–65 per cent of the total grey volume to neurons, 30 per cent to glial tissue, and 5–7 per cent to extracellular space (HORSTMANN and MEVES, 1959). Considerable functional importance obviously must be attributed to the neuropil.

These conclusions on the nature of the neuropil agree with the results obtained in analysing the SDA in it. The enzyme activity of the neuropil seems to show relations to both dendrites and glial tissue.

The blending of SDA in dendrites with the neuropil suggested that the distribution of enzymic activity in the neuropil is a function of dendrite branching. Preparations of cerebellar granular cells supported this concept showing SDA localized almost exclusively in the peripheral dendrite branchings which are accumulated in the glomerula cerebellaria; SDA was absent in the bodies of isolated granular cells and also was absent in isolated glial cells.

VOL.
5
1959/6

There were, on the other hand, indications of a relationship of glial tissue to the SDA in the neuropil, since there were twice as many glial cells present in the neuropil than in white matter. The absence of SDA in isolated glial cells could be misleading, since SDA is normally absent in the perikarya of Schwann cells, although it is very strong in the Schwann processes at the nodes of Ranvier (COHEN and ROMANUL, 1959; FRIEDE, 1959*c*). Glial cells, therefore, may contribute to the enzymic activity of the neuropil although no activity is found in their cell bodies. In this way, the interpretation of staining or isolation techniques may imply misleading conclusions, if the probability of biochemical differences between cell body and cell processes is not taken into account.

Neuropil may be defined as the close juxtaposition of dendrites, glial and axon branchings, including synapses, forming a complex tissue unit which may differ from the perikarya of the constituting cells in its histochemical properties and possibly also in its functional properties. Neuropil, thus, can be considered as a biological unit, although there is no doubt about its composition of heterogenous elements.

Inasmuch as dendrite branching contributes considerably to the formation of the neuropil, brain metabolism may be regarded chiefly as dendrite metabolism (LOWRY *et al.*, 1954). The perikarya of the numerous cells of the upper cortical layers, for example, seem to play a relatively small part in the total activity of the citric acid cycle of this region. This is not true for the fifth and sixth layer of the isocortex in which enzymic activity definitely prevails in the cell bodies. The relative increase of the cell's surface may be a determining factor for the metabolic activity of the neuropil (LUMSDEN, 1957).

The metabolic activity of the neuropil may be a critical factor for the vulnerability of brain tissue; for example, the vulnerability to anoxia of the individual areas of the dog's cerebral cortex decreased in the following sequence: 5, 7, 50, 17, 18, 4, 3, 6, 21, 22, 52 and 8 (FISCHER and PELESKA, 1957). This gradation reflects approximately the gradation of SDA in the neuropil of these areas. A patchy decrease of SDA in the neuropil has been observed in biopsies of pathological human cerebral cortex (FRIEDE, 1959*b*).

The mapping of enzymic activity of the cerebral cortex may bear neurophysiological implications. Investigations with microelectrodes have shown that an explanation of the slow brain rhythms by summation of individual synaptic potentials seems as unlikely as an explanation by summation of neuronal spikes, although both phenomena might contribute to the genesis of brain waves. Structures of large extent and mass effect had to be assumed to explain the extensive field and spread of brain potentials (JUNG, 1953).

A gradual reversal of the potentials of slow waves at the depth of about the fourth layer was recorded with microelectrodes whereas the slow waves of the fifth layer were independent from the simultaneous record from the cortical surface (LI and JASPER, 1953, postsigmoid gyrus of the cat). RENSHAW, FORBES and MORRISON (1940) recorded unitary discharges of pyramidal cells in the pyramidal stratum of the hippocampus but recordings from the surface showed only slow waves without suggesting any relationship to the spike discharges. Similar observations have been made on the cerebellar cortex (BROOKHART, MORUZZI and SNIDER, 1950). This behaviour of slow brain potentials bears surprising similarities to the distribution of enzymic activity in the neuropil. It would seem, therefore, that the present mapping of enzyme activity in the cerebral cortex could be used as a guide for investigations on its electrophysiology.

SUMMARY

Differences of intensity and distribution of succinic dehydrogenase activity were found among certain cytoarchitectonic areas of the cerebral cortex of the guinea pig. In the isocortex, enzyme activity prevails in the neuropil in the upper three layers but it prevails in the cell bodies in the fifth and sixth layers. Particularly strong enzymic activity is found in the neuropil of the second and third layers of the postcentral, occipital and supratemporal cortex; enzymic activity is mild in these layers in the parietal and the precentral motor cortex and it is weak in the insular and lower temporal cortex. An absence of histochemically distinguishable laminae is typical for the cingular cortex (area 23).

The distribution of enzymic activity in the allocortex shows a considerable variability of patterns among individual areas, such as a trilaminate pattern in the *retrolimbic cortex* (area 29), a bilaminate pattern with particular intensity of enzymic activity in the molecular layer in the *pyriforme cortex* (area 51); or a detailed histochemical architectonics, as in the olfactory bulb and the *cornu ammonis*.

A mapping of the cerebral cortex is based on gradations of enzymic activity. The distribution of enzymic activity in isolated cells and the relationship between glial cells and the enzymic activity of the neuropil were investigated in two additional studies.

REFERENCES

APATHY ST. V. (1897) *Mitt. zool. Sta. Neapel* **12,** 44.
BAUER K. F. (1938) *Z. mikr.-anat. Forsch.* **43,** 48.
BAUER K. F. (1943) *Z. ges. Neurol. Psychiat.* **176,** 265.
BAUER K. F. (1953) *Organisation des Nervengewebes und Neurencytium Theorie.* Urban u. Schwarzenberg, Wien.
BECK E. (1950) *Dtsch. Z. Nervenheilk.* **163,** 183, 214.
BERGER H. (1921) *Z. ges. Neurol. Psychiat.* **69,** 46.
BOK S. T. (1929) *Z. ges Neurol. Psychiat.* **121,** 682.

BONIN G. V. (1950) *Arch. Neurol. Psychiat., Chicago* **67,** 135.
BRAITENBERG V. and ATWOOD R. P. (1958) *J. comp. Neurol.* **109,** 1.
BRAUN F. (1951) Dissertation, Hannover.
BRODMANN K. (1909) *Vergleichende Lokalisationslehre der Grosshirnrinde.* Leipzig.
BROOKHART J. M., MORUZZI G. and SNIDER R. S. (1950) *J. Neurophysiol.* **13,** 465.
BURGEN A. S. V. and CHIPMAN L. M. (1951) *J. Physiol.* **114,** 296.
CAJAL R. (1906) *Studien über die Hirnrinde des Menschen V.* (Transl. Leipzig, Barth).
CHU L. W. (1954) *J. comp. Neurol.* **100,** 381.
COHEN R. B. and ROMANUL C. A. (1959) 35th *meeting Amer. Ass. Neuropath., J. Neuropath.*
DIXON K. C. (1954) *Quart. J. exp. Physiol.* **39,** 129.
EAYRS J. T. (1953) *Nature, Lond.* **172,** 403.
EAYRS J. T. and TAYLOR S. H. (1951) *J. Anat. Lond.* **85,** 350.
ECONOMO C. v. (1926) *Klin. Wschr.* **5,** 593.
FERRERO C. (1947) *Schweiz. Arch. Neurol. Psychiat.* **59,** 41.
FISCHER J. and PELESKA B. (1957) *Rozhl. Chir.* **36,** 253.
FLEXNER L. B. and FLEXNER J. B. (1946) *J. cell. comp. Physiol.* **27,** 35.
FRIEDE R. (1954) *Acta anat.* **20,** 290.
FRIEDE R. (1955*a*) *Arch. Psychiat. Z. Neurol.* **193,** 1.
FRIEDE R. (1955*b*) *Acta anat.* **25,** 65.
FRIEDE R. (1955*c*) *Z. Zellforsch.* **41,** 509.
FRIEDE R. (1958) *J. Histochem. Cytochem.* **6,** 347.
FRIEDE R. (1959*a*) *J. Neurochem.* **4,** 101, 111 (These are articles I and II of this series).
FRIEDE R. (1959*b*) *Virchows Arch.* **332,** 216.
FRIEDE R. (1959*c*) *Exp. Neurol.* **1,** 441.
GERLACH J. (1872) *Medic. Centralblatt* **1872,** 237.
HAMMARBERG (1895) *Studien über Klinik und Pathologie der Idiotie.* Upsala.
HAUG H. (1953) *Acta anat.* **19,** 60, 153, 239.
HAUG H. (1956) *J. comp. Neurol.* **104,** 473.
HAUG H. (1958) *Quantitative Untersuchungen an der Sehrinde.* Thieme, Stuttgart.
HAWKINS A. and OLSZEWSKI J. (1957) *Science* **126,** 76.
HELD H. (1927) *Mschr. Psychiat. Neurol.* **65,** 68.
HESS A. (1953) *J. comp. Neurol.* **98,** 69.
HESS A. (1955) *J. comp. Neurol.* **102,** 65.
HOEPKER W. (1951) *Z. Altersforsch.* **5,** 256.
HORSTMANN E. and MEVES H. (1959) *Z. Zellforsch.* **49,** 569.
JUNG R. (1953) *Symp. Third Internat. EEG Congr.*
KOELLIKER H. (1850) *Mikroskopische Anatomie* Bd. 2, p. 474. Leipzig.
KRIEG W. J. S. (1946) *J. comp. Neurol.* **84,** 221.
KUHLMANN R. E. and LOWRY O. (1956) *J. Neurochem.* **1,** 173.
LASHLEY K. S. and CLARK G. (1946) *J. comp. Neurol.* **85,** 223.
LI C. L. and JASPER H. (1953) *J. Physiol.* **121,** 117.
LOWRY O. H., ROBERTS N. R., LEINER K. Y., WU M., FARR L. and ALBERS R. W. (1954) *J. biol. Chem.* **207,** 39.
LUMSDEN C. E. (1957) In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 91. Pergamon Press, New York.
MUEHLMANN M. (1936) *Beitr. path. Anat.* **96,** 361.
NACHLASS M. M., TSOU K. C., SOUZA E., CHENG C. S. and SELIGMAN A. M. (1957) *J. Histochem. Cytochem.* **5,** 420.
NEUMANN K. H. and KOCH G. (1953) *Hoppe-Seyl. Z.* **295,** 35.
NISSL F. (1898) *Münch. med. Wschr.* **45,** 988, 1023, 1060.
NISSL F. (1903) *Die Neuronenlehre.* Jena.
ORTMANN R. (1957) *Acta anat.* **30,** 542.
OZZELLO L., LENDING M. and SPEER F. D. (1958) *Amer. J. Path.* **34,** 363.
REBHAN I. (1956) *Acta anat.* **27,** 361.
RENSHAW B., FORBES A. and MORRISON B. R. (1940) *J. Neurophysiol.* **3,** 74.
ROBINS E., SMITH D. E. and EYDT K. M. (1956) *J. Neurochem.* **1,** 54, 68, 77.

VOL.
5
1959/6

ROSE M. (1912) *J. Psychol. Neurol., Lpz.* **19,** 389.
SCHENK U. W. D. (1947) *Rev. d'Otol.* **19,** 42.
SHARIFF G. H. (1953) *J. comp. Neurol.* **98,** 381.
TOWER D. B. (1954) *J. comp. Neurol.* **101,** 19.
WINKLER C. and POTTER A. (1911). *An anatomical guide to experimental researches on the rabbit's brain.* Amsterdam.

Journal of Neurochemistry, 1960, Vol. 5, pp. 172–176. Pergamon Press Ltd. Printed in Northern Ireland

INTERRELATIONSHIPS BETWEEN ELECTROSHOCK, THE BLOOD-BRAIN BARRIER, AND CATECHOLAMINES*

S. ROSENBLATT, J. D. CHANLEY, H. SOBOTKA and M. R. KAUFMAN
The Depts. of Psychiatry and Chemistry, Mt. Sinai Hospital, New York, N.Y.

(*Received* 21 *October* 1959)

THE blood–brain barrier is a complex biological system which controls the exchange of substances between the plasma and the central nervous system. It consists of several component barrier systems: (a) the blood–cerebrospinal fluid barrier, (b) the cerebrospinal fluid–brain barrier, and (c) the blood–brain intercellular fluid barrier. The effect of these barriers on the transfer of solutes tends to be similar (HOLMES and TOWER, 1955).

Generally, the selective permeability of the barrier is altered only under relatively extreme conditions for the organism (BAKAY, 1956). However, it has been shown that an increase in permeability follows seizures induced by insulin, metrazol, and electric currents (HARTELIUS, 1952; PRADOS Y SUCH, 1936). The application of coagulation currents to the intact dura results in a disruption of the blood–brain barrier without any gross or microscopic changes in the brain (CAUDILL *et al.*, 1951). Electroshock treatments in cats cause an increase in the blood–brain barrier permeability to cocaine, which lasts for several days (AIRD *et al.*, 1956). The intravenous injection of adrenaline or noradrenaline results in arousal, anxiety, and an increase in the electrical activity of the posterior hypothalamus (PORTER, 1952). When adrenaline or noradrenaline is injected directly into the spinal fluid it causes drowsiness or anaesthesia (LEIMDORFER and METZNER, 1949; FELDBERG and SHERWOOD, 1954). By circumventing the blood–brain barrier excessive concentrations were reached in the cerebrospinal fluid which gave rise to inhibiting effects. Investigations into the roles of noradrenaline and serotonin in the brain indicate that these substances may be either neurohumors in the vegetative centres or may modify transmission in these areas (VOGT, 1954). These amines are found primarily in the diencephalic, mesencephalic, and bulbar representations of sympathetic activity.

Therefore, since electroshock does alter blood–brain barrier permeability and catecholamines do have a significant action on brain function, the purpose of this investigation was to study the effect of electroshock treatments on the permeability of the blood–brain barrier to catecholamines. This was done by estimating the levels of catecholamines in the cerebrospinal fluid: (a) before and after infusion of catecholamines into the blood, (b) before and after electroshock treatments, and (c) before and after a combination of electroshock and catecholamine infusion. Cerebrospinal fluid was used so that repeated studies could be made on the same dogs. The permeability changes in the blood–cerebrospinal fluid barrier component were taken as being analogous to changes in the rest of the blood–brain barrier system.

* This work was supported by a grant from the U.S. Public Health Service, National Institute of Health Grant M-1552. A preliminary report was presented at the American Psychiatric Association meeting San Francisco, May 1958.

VOL.
5
1959/6

EXPERIMENTAL

Spinal fluid was obtained by cisternal tap with the dogs under Nembutal (Abbott) anaesthesia. The spinal fluid was removed by allowing it to drip slowly from a pediatric spinal needle (22 gauge) into a small Erlenmeyer flask resting in an ice bath. The fluid was not withdrawn with a syringe because of the danger of rupturing blood vessels. An average of 5 ml could be obtained this way, depending on the size of the animal. Specimens which were not analysed immediately were placed in the deep freeze. If the tap was bloody, the procedure was discontinued, the specimen discarded, and the animal not used again for several days. Noradrenaline solutions were made up in isotonic saline just before use. Initially adrenaline as well as noradrenaline was used in the experimental procedures. However, when it became necessary to infuse dogs with relatively large amounts of catecholamines, the use of adrenaline was abandoned because of marked cardiac irregularities.

Intravenous infusions were given with conventional drip infusion apparatus (Venopak, Abbott). In some of the experiments, spinal fluid was collected over an entire hour, but in most instances it was collected at the end of an hour of infusion. Where the infusion ran continuously during the hour in which electroshocks were administered every 15 minutes, a small bore polyethylene tube was inserted into the vein instead of the usual intravenous needle. The rate of infusion was controlled as closely as possible by continuously observing the i.v. drip chamber and regulating the flow accordingly.

The ferricyanide oxidation method, with some modifications (see below), was used for the determination of noradrenaline (VON EULER, 1956). The fluorescence was measured with the Farrand photofluorimeter, Model A. The noradrenalutine fluorescence was activated at a wave length of 436 mμ, Corning filters 5113 and 3389 being used for the primary, and the emitted light was read through a Corning 3486 filter. The primary standardization of fluorimetric readings was obtained by oxidizing adrenaline and noradrenaline solutions of suitable concentrations as described below. However, in order to utilize fully the full range of sensitivity of the instrument, obviate the necessity of performing daily standard oxidations and correct for daily variations in the performance of the instrument, we correlated the fluorimetric readings obtained from the oxidation of adrenaline and noradrenaline with the fluorescence of at least three different standard quinine solutions made up in 0·1 N-H_2SO_4.

Autoxidation of catecholamines was controlled by using an ice bath and a refrigerated centrifuge in the extraction procedure. In order to minimize the precipitation of aluminum hydroxide when strong alkali is employed in the isomerization step, the chelating agent, Versene Fe 3 (Dow Chemical Co.) was used. Another source of error in estimating the lutine fluorescence came from fluorescent oxidation products of ascorbic acid which developed rapidly when the solution was made alkaline in the presence of Fe^{+++}. We therefore reduced the Fe^{+++} with glutathione before adding the ascorbic acid. The ascorbic acid and NaOH solutions were added separately. By the addition of Versene Fe 3 and the glutathione the sensitivity of the method was somewhat reduced, but reproducibility was considerably improved. We were able to estimate noradrenaline at a level of 5 mμg/ml.

Catecholamine Extraction. For the extraction of catecholamines, the cerebrospinal fluid specimen was transferred to a plastic centrifuge tube containing 700 mg of alumina (Woelm Alumina, non-alkaline, activity Grade 1, prepared according to COHEN and GOLDENBERG, 1957), 10 ml of 0·2 N-sodium acetate buffer (adjusted to pH 8·4 with dilute Na_2CO_3 solution) and 2 ml of 3% sodium thiosulphate. The solution was magnetically stirred in an ice-bath for 5 min while the pH was maintained between 8·1 and 8·5 by the addition of dilute Na_2CO_3 solution. After centrifugation, the supernatant solution was discarded, and the alumina washed twice with 5 ml portions of ice cold glass-distilled water. Catecholamines were eluted with 5 ml of 0·2 N-acetic acid, followed by 5 ml of water.

Catecholamine oxidation. Two ml of 1 M-acetic acid-acetate buffer of pH 6·0 were added to the above acid eluate followed by 0·5 to 1 ml of 3% Versene Fe 3, and the pH was adjusted to 6·5 with 0·1 N-NaOH. The solution was divided into two portions in 10 ml glass-stoppered cylinders. In one, the noradrenolutine fluorescence was eventually stabilized (S) while in the other the fluorescence was allowed to fade (F), and this served as the blank. The oxidation, tautomerization, and stabilization steps were carefully timed in order to control and equalize the spurious fluorescence in both solutions, which develops as a function of time. To the portion (F), 0·1 ml of 0·25% $K_3Fe(CN)_6$ solution was added, and oxidation was allowed to proceed for 3 min. When 2 min had elapsed, 0·1 ml of 0·25% $K_3Fe(CN)_6$ solution was added to the other portion, (S). At the end of the 3rd min 0·9 ml of 20%

NaOH solution was added to portion (F) and the fluorescence was allowed to fade for 3 min. At the end of the 5th min (portion (S) having been oxidized for 3 min) 0·3 ml of a 6% glutathione solution was added to (S) and the cylinder shaken until the yellow ferricyanide colour was discharged (10–15 sec). This was followed in rapid succession by 0·05 ml of 2% ascorbic acid and 0·9 ml of 20% NaOH solutions. The final solution was mixed thoroughly. These latter steps took about one min if the indicated amounts of the various solutions had previously been drawn into pipettes and placed to one side. At this point, the end of the 6th min, the lutine fluorescence in aliquot (F) had completely faded. Glutathione, 0·3 ml of a 6% solution, was added to (F), the mixture was shaken until the yellow ferricyanide colour disappeared, and 0·05 ml of 2% ascorbic acid solution was added. This tube (F) was used as the blank. Both glutathione and ascorbic acid solutions were made up daily. The final solutions were transferred to matched test tubes, and the fluorescence was read at 1, 5, 10, and 15 min. After 5–10 min the difference (S − F) remained constant. A control pre-treatment spinal fluid specimen was carried through the same steps outlined above.

RESULTS

Noradrenaline, if present in dog cerebrospinal fluid, did not exceed a concentration of 18 mμg/ml, which was the limit of sensitivity of the method (Table 1). When

VOL. 5 1959/6

TABLE 1.—LEVELS OF CATECHOLAMINES AND AN UNKNOWN COMPOUND IN CEREBROSPINAL FLUID OF DOGS UNDER VARIOUS CONDITIONS

Experimental conditions	No. of expts.	Unknown compound (arbitrary fluorescent units/ml)	Catecholamines (mμg/ml)
A. Control	4	0	0†
B. Adrenaline infusion of 5·3 μg/kg per min for 1 hr	4	0	0
C. Noradrenaline infusion of 4·8 μg/kg per min for 1 hr	4	1·7 (0·43)*	0
D. 5 electroshocks over 1 hr	4	0	0
E. 2 electroshocks followed by noradrenaline infusion of 9·8 μg/kg per min for 1 hr	6	2·5 (0·81)*	0
F. 5 electroshocks over 1 hr and simultaneous noradrenaline infusion of 9·8 μg/kg per min for 1 hr	4	2·8 (0·70)*	0
G. Noradrenaline infusion of 12·8 μg/kg per min for 1 hr in animals prepared with resperine and iproniazid‡	2	4·0 6·0	31·0 56·0
H. As in G, but preceded by 7 electroshock treatments over 10 days	2	4·0 5·0	120·0 181·0

* Standard deviation

† The limit of sensitivity of the method used was 18 mμg/ml of noradrenaline in cerebrospinal fluid.

‡ Preparation of animals in experiments G and H consisted of the following dosages give intravenously: reserpine 1 mg/kg on the first day; this was repeated the second day plus iproniazid 75 mg/kg. On the third day, several hr before the control specimen was taken and the noradrenaline infusion begun, iproniazid was repeated at the same dosage. Electroshock treatments were administered with the Medcraft ECT Unit, Model B-24, (Medcraft Electronic Corp., New York) at a dosage of from 130–150 V for 0·3–0·5 sec, sufficient to produce a grand mal seizure.

adrenaline or nonadrenaline was infused without special preparation of the animals other than intravenous anaesthesia, there was no elevation of the level of these substances in the cerebrospinal fluid (experiments B and C). Neither did catecholamines appear in the cerebrospinal fluid when electroshock treatments were administered as in experiment D, or when seven treatments were given over 10 days (not shown in the Table). In experiments C, E, and F, when noradrenaline was infused with or without electroshock treatment, an unexpected result was noted. The fluorescence of the faded solution of the post-treatment specimen was greater than that of the pre-treatment faded solution and greater than that of the post-treatment stabilized solution. The fluorescence of the unknown substance remained stable under the conditions where catecholamine lutine fluorescence faded, and did not develop in the same manner as the lutine fluorescence.

In experiments G and H, where reserpine and iproniazid (Marsilid, Hoffman-LaRoche, Inc.) were used, noradrenaline as well as the unknown substance were found in the spinal fluid. Also in experiment H, where the dogs received a course of electroshock treatment, there was about a threefold increase in noradrenaline in the spinal fluid over experiment G where no electroshock was given.

DISCUSSION

It has been suggested that the manner whereby reserpine exerts its tranquilizing effect (sometimes resulting in a frank depression) is by its interference with storage mechanisms binding noradrenaline and serotonin, thereby causing their release from brain depots (SHORE *et al.*, 1957). From the clinical observations that iproniazid, a monamine oxidase inhibitor, exerts a beneficial effect in the treatment of depression, and from the experimental evidence which indicates that this compound interferes with the destruction of brain noradrenaline and serotonin (SHORE *et al.*, 1957), it would follow that there is a relationship between this protective action of iproniazid and the increased activity of the arousal system in subcortical centres and brain stem.

The above evidence may be marshalled into a hypothesis relating the role of noradrenaline and/or serotonin in the central nervous system to the clinical psychiatric syndrome of the involutional or psychotic depression, i.e. the depressive state in which the so-called 'organic' component is quite marked. Thus, if one or both of these substances is involved in the mediation of the activity of the subcortical and brain stem centres regulating sympathetic arousal and alerting activity, then it can be expected that the turnover rate of the neurohumor will be a function of the state of activity of these centres, i.e. the extent to which the organism is in a state of heightened arousal. The psychological counterpart of this neurophysiological state is clinically recognized by the presence of fear and/or rage. The psycho-physiological syndrome of depression may be conceived as the outcome of a prolonged state of continuous emotional arousal in which the individual has been unable to make the necessary adaptations, psychologically, which would terminate the emergency state. The biochemical consequences of this perpetuated state of heightened emotional arousal may be a gradual depletion of one or more of the vegetative centre neurohumors if their rate of synthesis in, or restoration to (via the blood–brain barrier), the subcortical centres fails to keep pace with the rate of utilization. A depressive state may become psychologically irreversible when the noradrenaline concentration in the sub-cortical centres falls below some critical threshold value for the maintenance of adequate and

integrated activity. Clinically, depressed states which are amenable only to physiological or pharmacological therapy occur almost exclusively in the middle and older age groups, in which a decreased blood–brain barrier permeability may exist.

Electroshock treatment may mediate its effect on the physiology of depression by increasing the permeability of the blood–brain barrier to noradrenaline and/or serotonin as well as transiently raising their levels in the blood (WEIL–MALHERBE, 1955), thereby increasing the available supply of the neurohumors to the sub-cortical centres and brain stem. It has also been suggested (by the referee of this paper) that the observed increase in noradrenaline in the spinal fluid after administrstion of iproniazid and reserpine may, in fact, result from a direct effect on the permeability of the blood-brain barrier. This remains to be elucidated.

Preliminary studies on the nature of the unknown substance indicated that it was not the 3-methyl ether of noradrenaline, 3-methoxy-4-hydroxy mandelic acid, serotonin, or 5-hydroxyindoleacetic acid. The finding of presumably a new metabolite in the cerebrospinal fluid after intravenous infusion of noradrenaline suggests that there may be important alternative paths in the metabolism of catecholamines.

VOL.
5
1959/6

SUMMARY

We were able to demonstrate the permeation of noradrenaline through the blood-cerebrospinal fluid barrier when reserpine and iproniazid were used to prevent binding and oxidation respectively. Whether this was accomplished because higher blood levels were achieved, or because the noradrenaline which passed through the blood-brain barrier and the brain itself was spared, cannot be stated at this time.

When reserpine and iproniazid were not used, noradrenaline was not found in the spinal fluid but, unexpectedly, another substance made its appearance. It is not known at this time whether this substance is formed from noradrenaline peripherally and then penetrates the barrier or whether it is formed in the passage of noradrenaline through the barrier system or the brain itself. It could also be some unrelated substance which is released by noradrenaline. Electroshock treatment appears to alter the permeability of the barrier system to noradrenaline.

REFERENCES

AIRD R. B., STRAIT L. A., PACE J. W., HRENOFF M. K. and BOWDITCH S. C. (1956). *Arch. Neurol. and Psychiat., Chicago* **75,** 371.

BAKAY L. (1956) *The Blood–brain Barrier*. Thomas, Springfield, Ill.

CAUDILL C. M., SMITH G. A., FRENCH L. A., PEYTON W. T. and MOORE G. E. (1951) *J. Neurosurg.* **8,** 423.

COHEN G. and GOLDENBERG M. J. (1957) *Neurochem.* **2,** 71.

VON EULER U. S. (1956) *Noradrenaline*, Thomas, Springfield, Ill.

FELDBERG W. and SHERWOOD S. C. (1954) *J. Physiol.* **123,** 148.

HARTELIUS H. (1952) *Acta psychist., Kbh.* Suppl. 77.

HOLMES J. H. and TOWER D. B. (1955) *Neurochemistry*. Thomas, Springfield, Ill.

LEIMDORFER A. and METZNER W. R. T. (1949) *Amer. J. Physiol.* **157,** 116.

PORTER R. W. (1952) *Amer J. Physiol.* **169,** 629.

PRADOS Y SUCH M. (1936) *Arch. Psychiat. Nervenkr.* **105,** 17.

SHORE P. A., MEAD J. A. R., KUNTZMAN R. G., SPECTOR S. and BRODIE B. B. (1957) *Science* **126,** 1063.

VOGT M. (1954) *J. Physiol.* **123,** 451.

WEIL-MALHERBE H. (1955) *J. ment. Sci.* **101,** 156.

WEIL-MALHERBE H. and BONE A. D. (1952) *Biochem. J.* **51,** 311.

Journal of Neurochemistry, 1960, Vol. 5, pp. 177–184. Pergamon Press Ltd. Printed in Northern Ireland

THE EFFECTS OF HEATED SNAKE VENOM ON THE PHOSPHATE METABOLISM OF THE RAT SPINAL CORD

A. J. HUDSON, J. H. QUASTEL and P. G. SCHOLEFIELD*
McGill-Montreal General Hospital Research Institute, 3619 University Street, Montreal

(*Received* 3 *September* 1959)

THE intrathecal injection of agents, such as ^{32}P, in investigations on phosphate turnover in the brain, has become a well established procedure. Radioactive phosphate, when injected by routes other than the intrathecal, is only slowly taken up by the nervous system (HEVESY and HAHN, 1940; SAMUELS *et al.*, 1951) even with high plasma levels of ^{32}P, but the intrathecal injection into the cisterna magna leads to rapid uptake by the brain (BAKAY and LINDBERG, 1949; SACHS and CULBRETH, 1951). LINDBERG and ERNSTER (1950) have shown that the injected phosphate is rapidly incorporated into organic phosphates such as adenosine triphosphate.

The most suitable site of injection for experiments with spinal cord is the intrathecal space where agents may be administered in direct contact with the cord without mechanical injury. Heated *Naia Naia* cobra venom, as a source of phospholipase A has been injected (in experiments to be described in this paper) into the spinal canal of rats in order to observe the physiological effects of the heated venom. This procedure avoids complication due to interference by the blood-brain (spinal cord) barrier.

The toxic effect of snake venom on the nervous system was demonstrated by FLEXNER and NOGUCHI (1902). Snake venom contains many pharmacologically active substances of which proteins with enzymic activities constitute a large part. WEIR-MITCHELL (1860) discovered that venom, when heated to 100° for 30 min, retained its toxicity, and in a recent study BRAGANCA and QUASTEL (1953) observed that when cobra venom solution was heated for 15 min at 100°, and the toxicity examined by intramuscular injection in mice, 50 μg of the heated venom caused death in 4 hr. The neurotoxic factor is possibly identical with the relatively heat stable enzyme, phospholipase A.

The effect of venom in producing demyelination was observed by MORRISON and ZAMECNIK (1950) who incubated rabbit spinal cord with venom and examined the tissue histologically. The venom was presumed to act by the removal of a molecule of fatty acid from lecithin by the venom phospholipase, leaving lysolecithin. Lysolecithin itself is a potent haemolytic and possibly lypolytic agent.

PETRUSHKA, QUASTEL and SCHOLEFIELD (1956, 1959*a*,*b*) have observed that heated venom (phospholipase A) affected the respiratory activity of brain slices but not that of liver slices. The effect on brain slices was to increase respiratory activity for a short time and then to diminish it.

The following investigation deals with the effects of heated snake venom on the

* Research Associate, National Cancer Institute of Canada.

respiration of spinal cord slices and the ^{32}P incorporation into various fractions of spinal cord.

METHODS AND MATERIALS

Intrathecal injections with ^{32}P

Hooded rats of an average weight of 265 (±25) g were lightly anaesthetized with chloroform followed by the intraperitoneal injection of 5 mg of nembutal. The overall period of anaesthesia was approximately 30 min. The dorsum of the rat over the lumbar and lower thoracic regions was shaved and a longitudinal incision 1 cm in length was made over the spinous processes at the prescribed site of injection (*vide infra*). The subcutaneous tissue was resected in order to expose the spinous processes, and the anaesthetized rat placed across a wooden block designed to produce a degree of spinal flexion which would facilitate the insertion of a needle between the spinous processes into the spinal canal (Fig. 1, a and b). The injected material presumably enters the spinal canal near the junction of the lumbar and thoracic regions.

A three-quarter inch, 26 gauge needle attached to a tuberculin syringe containing heated venom or ^{32}P sodium phosphate (2–3 × 10^6 counts/min) in a neutral solution of isotonic saline was inserted between the spinous processes at a point approximately 6·5 cm from the ischial tuberosities (or approximately 9 cm from the foramen magnum). With the needle thus inserted into the spinal canal, a volume of 0·05 or 0·1 ml was injected with as little force as possible and care was taken to ensure that all the injected solution entered the intrathecal space. The complete entry of the injected solution into the intrathecal space is determined largely by the ease with which the injection is made since in animals of this size it is not possible to withdraw spinal fluid. No injury to the spinal cord occurred when the site of injection lay within a prescribed limit as noted above, i.e. below the tip of the conus medullaris.

Removal of spinal cord and isolation of tissue fractions

The rats were decapitated at various intervals up to 16 hr following injection of ^{32}P, the entire vertebral column was resected, and the intact spinal cord removed by dissection. The cord was washed for 15 sec in running water, transversely cut into three portions which corresponded to cervical, thoracic, and lumbar regions, weighed, and each section placed in a tube containing 5 ml of cold 5% trichloroacetic acid (TCA) solution.

Each section of the cord was homogenized, kept at 4° for 1 hr, and centrifuged. The supernatant fluid was removed, and the residue resuspended in 5 ml of cold 5% TCA. The supernatant fluid, following a second centrifugation, was added to the first, and a sample plated and counted (TCA soluble fraction). The procedure was repeated a third time and the supernatant fluid was discarded. The residue was resuspended in 5 ml of 80% alcohol and kept at 50–60° for 30 min. After centrifugation, the supernatant fluid was removed, and the residue resuspended in 5 ml of equal parts of 80% alcohol and ether. This was kept at 40°–50° for 15 min, the supernatant fluid removed and added to the alcohol fraction. A portion was plated and counted (phospholipid fraction). The residue was dissolved in 1 ml of M-KOH and left overnight. A portion was added to a planchet containing a slight excess of 15% TCA, taken to dryness, and counted ('insoluble fraction').

The TCA soluble and the alcohol, alcohol–ether-soluble fractions of the three regions of the cord were each plated in 0·2 ml quantities, and the total counts of each fraction determined with an end-window type counter. The results were calculated as counts/minute per mg of wet weight for the three regions and for the whole cord.

The TCA-soluble fraction was further treated by the addition of 10 ml of ether, the mixture thoroughly agitated, and the ether removed. This was repeated three times to remove all the TCA, and a portion (approximately 1 ml) was removed and evaporated to dryness. A few drops of distilled water were added to the residue, the solution was transferred to paper and subjected to two dimensional chromatography, radioautographs were made, and the chromatograms counted for the 'inorganic' and 'ATP–ADP' fractions according to the method described by CREASER, DE LEON and SCHOLEFIELD (1959).

The results obtained for the extent of labelling of the inorganic phosphate fraction require a minor correction owing to the presence of some surface contamination with ^{32}P of the sections of the spinal cord. The procedure, however, should ensure that all organic phosphate fractions, such as the 'ATP-ADP' fraction, have been freed of contamination with radioactive inorganic phosphate.

VOL. 5 1959/6

Fig. 1a

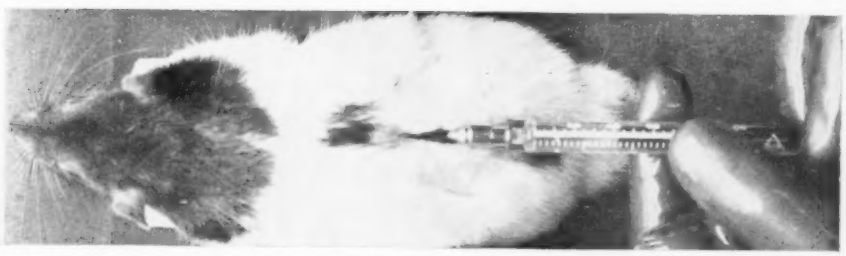


Fig. 1b

FIG. 1a and b.—Demonstration of method of intrathecal injection into the rat.

VOL.
5
1959/6

Preparation of heated snake venom

Naia Naia cobra venom (Indian cobra) was prepared by heating a solution for 15 min in a boiling water bath, and the precipitated protein removed by centrifugation. The supernatant fluid was decanted into a clean vessel, made isotonic with NaCl (0·9%), and kept at 4° until required. The appearance of the preparation was opalescent and a further fine precipitate usually appeared subsequently; no attempt was made to remove this. A standard preparation of 125 μg (weight prior to heating) in 0·05 ml was used for the intrathecal injections.

Incubation procedure with spinal cord slices

Slices of rat spinal cord, the lumbar cord only being used, were prepared as follows: hooded rats of an average weight of 265 g ($\pm$25 g) were used in the experiments. Immediately following a blow on the head the rats were decapitated and the vertebral column from the atlas to the mid-lumbar region was removed by dorsal resection. The vertebral column was transversely cut into four parts, and the cord removed from the lumbar section by passing a tightly fitting blunt probe along the vertebral canal. The probe, when rotated and gently forced along the canal, tears the denticulate ligaments and nerve roots with very little injury to the spinal cord with the result that short lengths of intact cord are obtained. The most caudal portion containing the *cauda equina* was discarded. Each whole piece of cord thus obtained was placed immediately on ice and sectioned once through the longitudinal axis with a Stadie-Riggs tissue slicer. During sectioning the tissue was gently pressed with the result that two flat pieces were obtained. Although they were of excessive thickness (0·3–0·9 mm) attempts at further slicing usually severely traumatized the tissue. The two halves were incubated together so that the results obtained reflect the activity of a whole region of the spinal cord.

The standard Warburg manometric technique was adopted for incubation. The slices were placed in Krebs-Ringer phosphate medium containing 0·01 M-glucose and 2·5 mg of heated cobra venom as required, ^{32}P phosphate being placed in the side-arm. After oxygenation for two minutes an equilibration period of 10 min at 37° was allowed. Radioactive phosphate was tipped from the side-arm and after a further equilibration period of 4 min the slices were incubated for 90 min.

RESULTS

The uptake and incorporation of intrathecally injected ^{32}P in spinal cord

Of a solution containing ^{32}P, 0·1 ml was injected into the lumbar-thoracic intrathecal space of rats. The rats were decapitated after various intervals of time and the ^{32}P present in the total acid-soluble and the phospholipid fractions of the cervical, thoracic and lumbar regions was estimated. The results (Table 1) show that there occurs a rapid diffusion of ^{32}P up the spinal canal. When a smaller volume was injected (0·05 ml) the uptake in the cervical region, when compared with that of the thoracic and lumbar regions, was proportionately less than that observed in this series.

There is a rapid uptake of ^{32}P into the TCA-soluble fraction (Table 2). In the rat, decapitated at 3 min after injection, there was already considerable uptake in the cold TCA fraction and incorporation into the phospholipids (alcohol, alcohol–ether fraction). However, the time required for the complete removal of the cord and placing in TCA was 20 min and in this period there is probably extensive uptake and incorporation of ^{32}P into the cord. When, for example, the maximal uptake occurs in the rat (decapitated 15 min after injection) either a part or whole of the time required for dissection must be taken into consideration. Thus, a maximal uptake most probably occurs not later than 35 min after injection, although an accurate estimate cannot be made. The incorporation of ^{32}P into the phospholipid fraction is already evident in the rat decapitated 3 min after injection of ^{32}P.

After attaining a maximal value, the ^{32}P content of the acid soluble fraction shows a steady but gradual decline. However, the incorporation of ^{32}P into the phospholipid

TABLE 1.—UPTAKES OF INTRATHECALLY INJECTED ^{32}P* AT DIFFERENT LEVELS OF THE RAT SPINAL CORD (TRICHLOROACETIC ACID SOLUBLE FRACTION)
(*counts/min per mg of wet wt. of tissue*)

Time	Spinal cord level		
	Cervical	Thoracic	Lumbar
3 min	1378	2780	2240
15 min	1090	4838	2935
30 min	1383	4730	1839
1 hr	1980	4565	2065
2 hr	1558	3196	3060
3 hr	1805	4088	2420
4 hr	2020	3588	2495
5 hr	1705	3645	2665
6 hr	2175	3585	2340
16 hr	858	3450	2595

* $2{\cdot}3 \times 10^6$ counts/min (0·1 ml).

TABLE 2.—UPTAKE OF INTRATHECALLY INJECTED ^{32}P* INTO WHOLE RAT SPINAL CORD

Time	counts/min per mg wet wt of tissue	
	Cold trichloroacetic acid fraction	Alcohol, alcohol–ether fraction
3 min	2,079	50
15 min	2,802	103
30 min	2,713	147
1 hr	2,715	187
2 hr	2,525	382
3 hr	2,670	537
4 hr	2,635	653
5 hr	2,552	—
6 hr	2,588	781
16 hr	2,219	944

* $2{\cdot}3 \times 10^6$ counts/min (0·1 ml).

fraction increases steadily over the 16 hr period of the series with no evidence of a plateau or decline (Table 2). The turnover rate in the phospholipid compared with that in the acid soluble fraction is relatively small, although the early appearance of ^{32}P in this fraction would appear to indicate a fairly active incorporation into the high energy phosphate moiety.

VOL. 5 1959/6

The effects of heated venom on the metabolism of spinal cord slices

The effects of heated snake venom on the respiration of rat brain cortex slices (PETRUSHKA *et al.*, 1959*a*) and on the phosphate metabolism of mitochondria (PETRUSHKA *et al.*, 1959*b*) have been described. It was of interest to investigate the action

TABLE 3.—EFFECTS OF HEATED COBRA (*Naia Naia*) VENOM ON THE RESPIRATION OF RAT SPINAL CORD SLICES

Time (min)	Q_{O_2}	
	No venom	2·5 mg heated venom
0–15	6·6	6·4
15–30	6·0	5·8
30–45	5·2	4·75
45–60	5·1	4·5
60–75	4·8	4·2
75–90	4·8	3·5

The incubations were carried out 3 ml Krebs-Ringer phosphate medium containing 0·01 M-glucose at 37° under aerobic conditions. The heated venom was added from the side-arm at zero time.

TABLE 4.—THE EFFECTS OF HEATED COBRA (*Naia Naia*) VENOM ON THE PHOSPHATE TURNOVER OF SPINAL CORD SLICES

Fraction	^{32}P uptake counts/min per mg wet wt of tissue	
	No venom	2·5 mg heated venom
Inorganic phosphate	$1{\cdot}76 \times 10^4$	$1{\cdot}74 \times 10^4$
ATP + ADP	291	108
Phospholipids	227	126
Insoluble fraction	430	229

34·5 μmoles of phosphate were added (specific activity = $1{\cdot}51 \times 10^6$ counts/min per μmole). Other conditions as in Table 3.

The results are the mean of three separate determinations made under the same experimental conditions and calculated as ^{32}P taken up in 90 min (counts/min per mg wet weight of tissue).

of heated *Naia Naia* venom on the respiration of spinal cord slices. The results (Table 3) indicate that the normal fall in respiratory activity is accelerated by the presence of the heated venom.

When slices of spinal cord were incubated with ^{32}P-phosphate and the various phosphorus containing fractions isolated, the results shown in Table 4 were obtained. Different rates of incorporation of ^{32}P into the various fractions are found in the presence of venom, the value for the 'ATP–ADP' fraction showing the greatest change. In

the presence of the heated venom, the incorporation of ^{32}P into the 'ATP–ADP' fraction is only 37 per cent of the control value. The incorporations of ^{32}P into the 'phospholipid' and 'insoluble' fractions were 55 and 53 per cent of the corresponding control values, respectively.

The effect of intrathecally injected heated venom

An attempt was made to correlate the effects on the metabolism of spinal cord *in vitro* due to the addition of heated snake venom with possible *in vivo* reactions following intrathecal administration of the heated venom. When 125 μg of heated venom were injected into the lumbar intrathecal space, profound paralysis of the hindquarters of the rat was evident after its awakening from anaesthesia, approximately 15 min after the injection. The rat was obviously experiencing severe pain in the hindquarter region. Simultaneously there was evidence of hyperaesthesia changing to anaesthesia in the same region over the next 30 min to 1 hr. There was also incontinence of bladder and the development during the following week of a large cutaneous ulcer (probably trophic), at the site of injection.

After 3–4 weeks there was considerable improvement. The animals were able to walk, although unsteadily, incontinence disappeared, and the large ulcer on the dorsum healed.

When the amount of heated venom injected was increased, the mortality rate rose and with the administration of one or more milligrams the mortality rate became 100 per cent. The extent of paralysis could be varied to a certain extent by injecting larger or smaller amounts of venom.

DISCUSSION

BAKAY and LINDBERG (1949) have found that the uptake of intracisternally injected ^{32}P-phosphate into the brain occurs with considerable rapidity, a maximal concentration being attained in 30 min. The uptake of intrathecally injected ^{32}P-phosphate into spinal cord is similarly very rapid, the length of time required for removal of the cord making it difficult to state the actual time at which the peak concentration is attained, but it certainly occurs within 35 min. During this period there is active incorporation into the phospholipid fraction despite the anaesthetized state of the rat which ANSELL and DOHMAN (1957) have shown will depress phosphate turnover in phospholipids. The rats were only lightly narcotized in an attempt to minimize this factor.

Upon attaining a peak, the concentration of ^{32}P in the spinal cord falls slowly and 1 week later the concentration may be a third to a half of the maximal level. NAKA *et al.* (1954) reported that, 1 week after the cisternal injection of ^{32}P, the concentration in the brain had only decreased to one half the maximal level. Despite a high turnover rate, phosphate is held within the nervous tissue. This behaviour of phosphate is of interest in view of the poor transfer of phosphate ions from plasma. BAKAY (1957) has pointed out that, once in the nervous system, most substances stay longer than in any other parenchymatous organ. Certain structures in the nervous system show a rapid uptake and loss of ^{32}P, *e.g.* the *tuber cinereum*, *supraoptic crest*, *area postrema* in the medulla, and the *subfornical* regions (BAKAY, 1952), similar to the occurrence in other organs, *e.g.* liver and intestine (PERLMAN, RUBEN and CHAIKOFF, 1937).

It has been shown by STRICKLAND (1952) that ^{32}P is rapidly incorporated into the phospholipid and phosphoprotein of brain. The rise in ^{32}P in the phospholipid fraction

VOL. 5 1959/6

in the present experiments was continuous up to 24 hr but no further analyses were made. NAKA (1957) found that equilibrium between the various ^{32}P containing fractions was attained by the third day after intrathecal injection of ^{32}P.

In their studies of snake venoms, DELEZENNE and LEDEBT in 1911 correlated the presence of lecithinase with haemolysis and HOUSSAY, MAZZOCCO and NEGRETTE in 1922 suggested that the nervous system may be another site of action of this enzyme. The vulnerability of the nervous system, apart from the curare-like effects of venom on neuromuscular junctions, has been demonstrated by the respiratory paralysis which follows the administration of cobra venom directly into the brain near the bulbar centres (FRASER and ELLIOTT, 1905).

The effect of addition of heated venom (*e.g.* 2·5 mg) to spinal cord slices is to produce eventually an inhibition of respiration. No initial stimulation of respiration such as that seen with brain slices (PETRUSHKA, QUASTEL and SCHOLEFIELD, 1956, 1959*a*, *b*) is observed. In the presence of the heated venom the fall in respiratory activity of rat spinal cord is not as great as the reduced incorporation of ^{32}P in the 'ATP–ADP' fraction. This result probably reflects a decrease in the P : O ratio. In similar experiments with liver and kidney mitochondria a fall in the P : O ratio has actually been demonstrated and this has been interpreted as being due to an interference with phosphorylation mechanisms occurring before the interference with respiration (PETRUSHKA, QUASTEL and SCHOLEFIELD, 1956, 1959*a*, *b*). BRAGANCA and QUASTEL (1953) in a study of the inhibitory action of heated venom on a variety of enzyme systems present in homogenates suggested that the effect may be exerted 'by enzymic degradation of mitochondrial structures upon whose integrity or particular spatial configuration the enzyme systems concerned depend for their optimal activities'.

The effect of the venom on the metabolism of cord slices is not as striking as would be expected from the swiftness and severity of paralysis observed with intrathecal injection of the venom. The initial site of action of venom may be on a constituent of the neuronal membrane, thus affecting metabolic activities closely connected with it (QUASTEL, 1957). Such an effect on the nervous system in the living rat would interfere with nerve impulse transmission and produce prompt paralysis before other activities of the cell were significantly affected.

The administration of 4–8 μg/gm body wt of heated venom, intraperitoneally or intrathecally, was found to be lethal to a rat, death occurring with no clinical evidence of central nervous system involvement, apart from paralysis. It should be noted, however, that heated venom *in vivo* produces lesions in the nervous system (SANDERS, AKIN and SORET, 1954).

SUMMARY

(1) A technique for the intrathecal injection of substances into rats is described. Radioactive phosphate was administered by the intrathecal route and its uptake into the cold trichloroacetic acid soluble fraction and alcohol, alcohol–ether fractions at various intervals following injection was studied.

(2) The uptake of ^{32}P into rat spinal cord *in vivo* was similar to that reported elsewhere for brain. The uptake into the cold trichloroacetic acid fraction is rapid, a peak concentration being attained within the first 35 min, followed by a gradual fall. The uptake into the phospholipid fraction occurs more slowly but continues to rise over a 24 hr period of observation.

(3) Heated cobra venom, when administered to rats in non-lethal amounts into the intrathecal space, produces a swift and severe paralysis from which there is considerable recovery. Initially the rats show pain, severe paralysis in the hindquarter region and incontinence. After 3 to 4 weeks they are able to walk, although unsteadily, and incontinence disappears.

(4) When spinal cord slices are incubated with heated cobra venom a fall in the respiratory activity is observed over a 90 min period. The incorporation of ^{32}P at 90 min in the 'ATP–ADP' fractions in the presence of venom is considerably smaller than that in the controls to which no venom has been added. The 'phospholipid' and the 'insoluble' fractions show a reduced turnover of ^{32}P although not to the extent observed in the 'ATP–ADP' fraction. The net effect of the presence of the heated cobra venom is a fall in the P : O ratio brought about by the activity of the phospholipase present.

(5) Although the effect of heated venom on the phosphate turnover of spinal cord slices is fairly marked, the changes are not striking when compared with the rapid onset of paralysis seen *in vivo*. It is suggested that the heated venom acts initially upon the nerve cell membrane, affects cell energetics by change in the P : O ratio, and produces paralysis by interfering with nerve impulse transmission.

VOL.
5
1959/6

Acknowledgement—This work was supported by a fellowship to one of us (A. J. H.), and by a research expense grant from the Multiple Sclerosis Society of Canada. The authors are greatly indebted to Dr. D. McRAE and Dr. H. JASPER of the Montreal Neurological Institute who provided facilities for *in vivo* radioisotope experiments.

REFERENCES

ANSELL G. B. and DOHMAN H. (1957) *J. Neurochem.* **2**, 1.
BAKAY L. (1952) *Arch. Neurol. Psychiat. Chicago* **68**, 629.
BAKAY L. (1957) In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 136. Pergamon Press, London.
BAKAY L. and LINDBERG O. (1949) *Acta physiol. scand.* **17**, 179.
BRAGANCA B. M. and QUASTEL J. H. (1953) *Biochem. J.* **53**, 88.
CREASER W., DE LEON R. and SCHOLEFIELD P. G. (1959) *Cancer Research* **19**, 705.
DELEZENNE C. and LEDEBT S. (1911) *C. R. Acad. Sc. U.R.S.S.* **152**, 790.
FLEXNER S. and NOGUCHI H. (1902) *J. exp. Med.* **6**, 279.
FRASER R. H. and ELLIOTT T. R. (1905) *Proc. Roy. Soc.* B. **74**, 104.
HEVESY G. and HAHN L. (1940) *K. danske vidensk. Selsk. Biol. Med.* **15**, No. 7.
HOUSSAY B. A. MAZZOCCO P. and NEGRETTE J. (1922) *C. R. Soc. Biol., Paris* **11**, 823.
LINDBERG O. and ERNSTER L. (1950) *Biochem. J.* **46**, 43.
MORRISON L. R. and ZAMECNIK P. C. (1950) *Arch. Neurol, Psychiat., Chicago* **63**, 367.
NAKA S. (1957) In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 297. Pergamon Press, London.
NAKA S., KATO Y., MUKAI A., AKIYAMA S., MATSUMOTO K. and MATSUMOTO S. (1954) *Kyushu Mem. med. Sci.* **5**, 51.
PERLMAN I., RUBEN S. and CHAIKOFF I. L. (1937) *J. biol. Chem.* **122**, 169.
PETRUSHKA E., QUASTEL J. H. and SCHOLEFIELD P. G. (1956) *Canad. Cancer Conf.* **2**, 106.
PETRUSHKA E., QUASTEL J. H. and SCHOLEFIELD P. G. (1959*a*) *Canad. J. Biochem. Physiol.* **37**, 975.
PETRUSHKA E., QUASTEL J. H. and SCHOLEFIELD P. G. (1959*b*) *Cadad. J. Biochem. Physiol.* **37**, 989.
QUASTEL J. H. (1957) In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 267. Pergamon Press, London.
SACHS J. and CULBRETH G. C. (1951) *Amer. J. Physiol.* **165**, 251.
SAMUELS A. J., BOYARSKY L. L., GERARD R. W., LIBET G. and BRUST M. (1951) *Amer. J. Physiol.* **164**, 1
SANDERS M., AKIN B. A. and SORET M. G. (1954) *Acta neuroveg.* **8**, 362.
STRICKLAND K. P. (1952) *Canad. J. Med. Sci.* **30**, 484.
WEIR-MITCHELL S. (1860) *Smithsonian Contrib. to Knowledge* **12**, 1866.

Journal of Neurochemistry, 1960, Vol. 5, pp. 185–194. Pergamon Press Ltd. Printed in Northern Ireland

CYTOPLASMIC pH OF NERVE FIBRES

C. S. SPYROPOULOS
Laboratory of Neurophysiology
National Institute of Neurological Diseases and Blindness
Bethesda, Maryland, U.S.A.

(*Received* 9 *February* 1959)

AFTER it became apparent that the concentration of hydrogen ions strongly affects many biological processes or properties of biological materials (e.g. enzyme rates, states of colloids and other amphoteric electrolytes), many attempts were made to determine the pH of extracellular and intracellular fluids of plants and animal systems. In some instances, the study of the hydrogen ion concentration of certain extracellular fluids such as blood, urine, or digestive juices has been very successful and the results have proved to be of immense biological and clinical value. The study of the intracellular pH has not met with equal success. This is so despite the fact that at one time or other it has been implicated in some of the most important biological phenomena (e.g. fertilization of cells, cleavage of cells, plant tropism, muscle contraction, nerve excitation, certain types of diseases, and death).

One of the most reliable techniques for the determination of pH is that in which the glass electrode is used. CALDWELL (1958) has recently applied this method to a very extensive study of the internal pH of muscle and nerve fibres. Many of the results obtained have far reaching implications. The present author has applied the glass electrode technique, independently of CALDWELL, to the study of axoplasmic pH. This paper is a report of these experiments.

The expression 'axoplasmic pH' as employed in this paper, will simply mean the pH which corresponds to the electromotive force given by a cell composed of a reference (calomel) electrode, the axoplasm, and an electrode (glass) responsive to hydrogen ions. (For information on the modern outlook on pH, however, see the paper 'British Standard 165: 150' published by the British Standards Institute, and the American Letter Circular LC993 of the U.S. National Bureau of Standards.)

The literature in the field of the pH of cytoplasm is voluminous. No attempt will be made to review it here. For the older literature one may refer to the reviews of REISS (1926), GRÄFF and RAPPOPORT (1937), LISON (1941), CHAMBERS (1949) and HEILBRUNN (1952). The more recent work has been very aptly covered by WIERCINSKI (1955), SMALL (1955) and CALDWELL (1956).

METHODS

Animal and tissue preparations. The giant axon of the stellar nerve of *Loligo pealii* was used. As a rule, the axons were not extensively cleaned. In some cases, the fibres and connective tissue surrounding the axon were carefully cleaned under darkfield illumination with dissecting needles in a manner comparable to that employed in dissecting vertebrate single nerve fibres (TASAKI, 1953). The diameter of the axons employed ranged from 350 to 650 μ. A total of 235 axons was employed. Of these, 176 were used for studies in which it was desired to collect axoplasm in relatively large quantities. In one series of experiments, the stellar and fin nerves were excised together, with care to keep the epineurium intact.

Preparation of electrodes. The material used for pH electrodes was Beckman's general purpose pH glass. Glass electrodes of this type require no sodium correction over the pH range of 1–11 and at room temperature. The glass was drawn down to a capillary of 100–200 μ in diameter and 10–12 cm in length. These tubes were stored in acid. Later, one end of the tube was sealed by fusing. The interior of the tube was filled with 0·1 N-HCl after extensive washing. This glass tube was made concentric with a much larger tube also filled with 0·1 N-HCl in such a manner that the HCl at the open end of the small tube made fluid contact with the HCl of the larger tube, and also in such a manner that 90 per cent of the length of the small tube protruded out of the end of the larger tube (Fig. 1, left). With the exception of the terminal 1–1·8 cm of the protruded glass tube, the remaining portion was surrounded with a suitable insulation such as paraffin, or sticky wax, or a combination

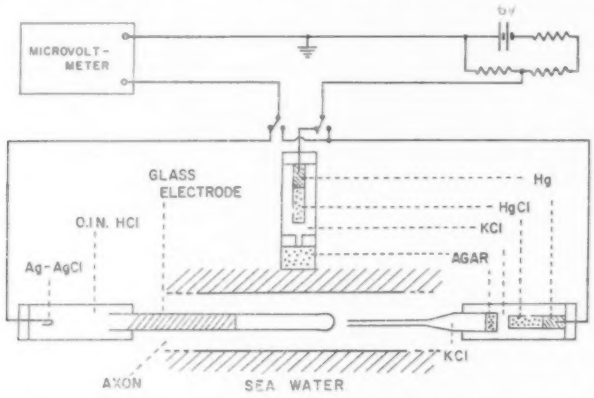


FIG. 1.—Diagram of arrangement for recording intracellular pH with microglass pH electrode.

of both, or, in rare instances, with 'formvar', or other such insulator. In the end of the large outer tube was sealed a short piece of silver wire by fusing it across a glass septum. This wire established contact between the 0·1 N-HCl in the large tube and the lead wire on the outside. The portion of the wire immersed in the acid was chlorided.

Once the glass electrode was prepared (Fig. 1, left), the terminal uninsulated, portion was never allowed to dry out. The outside was kept permanently in contact with distilled water or a buffer of pH 7·0.

Two types of calomel reference electrodes were used; a micro-calomel electrode and macro-calomel electrode (Fig. 1). In the macro-calomel electrode (Fig. 1, middle), the saturated KCl made contact with agar in sea water. In the micro-calomel electrode (Fig. 1, right), the saturated KCl made contact with agar in isotonic KCl which in turn made contact with isotonic KCl. With the micro-calomel electrode, the isotonic KCl and the agar–isotonic KCl were contained in a pipette whose tip diameter was 50–100 μ. In a few experiments, the pipette of the micro-calomel electrode was also filled with saturated KCl.

The potential difference between the macro-calomel and micro-calomel electrodes, once aged, was less than 0·1 mv and over long periods of time remained constant. The agar was employed with both electrodes to retard the mixing of saturated KCl with either the sea water outside the macro-calomel or the isotonic KCl in the salt bridge of the micro-calomel. In order to standardize the glass electrode as well as to determine whether or not the behaviour of the glass electrode was correct, a number of checks were performed. One was to find out whether or not the potential of each varied linearly with pH at a slope of 0·059 v per pH unit at 25°, or at a corresponding potential at a different temperature. Potential measurements were made in various standard buffers of known pH, as follows: acetate (sodium and potassium) buffers of pH 3·6, 4·0, 4·6 and 5·0 (WALPOLE, 1914); phosphate (sodium and potassium) buffers of pH 6·0, 6·4, 6·8, 7·0, 7·4 and 7·6 (SORENSON, 1912; CLARK and LUBS, 1916); borate–KCl–NaOH buffers of pH 8·0, 8·5, 9·0 and 9·5 (CLARK and LUBS, 1916); carbonate–bicarbonate (sodium) buffers of pH 10·0 (OSTROW and RUSSELL, 1951); potassium–hydrogen–phthalate buffer of pH 3·0 (CLARK and LUBS, 1916); the BECKMAN standard of pH 7·0; and the

FISHER standard of pH 8·0. All non-commercial buffers were made up at 0·05 M. Any electrode whose slope deviated from the theoretical by more than 2 per cent was not employed. Any electrode whose slope, although theoretically correct, still showed a varying potential difference for a given buffer at different times was discarded. Any electrode which, although showing a theoretical slope, still had individual readings that were off by more than 2 mv was discarded. Any electrode which showed a great lag in the response time was discarded.

The resistance of the electrodes employed (for 1 cm length of exposed tube) was usually found to be of the order of 800–1300 megohms. Because of this very high resistance, care was taken in construction of the holders for the electrodes, for the solutions and for the axonal preparation in order to avoid problems of shunting. For the same reason, care was taken to assure that the resistance of the

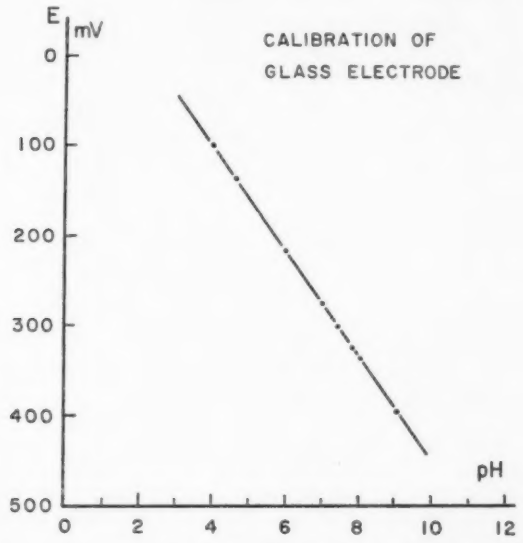


FIG. 2.—Typical calibration curve of microglass pH electrode. The buffer at pH 4·0 and 4·6 was acetate; at pH 6·0, phosphate; at pH 7·0, Beckman Standard and my own preparation of phosphate; at pH 7·5, phosphate. The buffers of higher pH were all borate. Temperature was 21°C. Length of uninsulated pH glass was 12 mm. The diameter of glass pH electrode was 150 μ. The resistance of the glass electrode was 1300 megohms.

insulated (with paraffin, sticky wax, etc.) portion of the glass electrode was sufficiently high. In this connection, tests were also carried out to make certain that the insulated portion was not pH sensitive. When an electrode showed a non-uniformity of pH sensitivity along the bared portion, it was discarded.

The calibration curve for one of the electrodes used is displayed in Fig. 2. The temperature was 21° and the resistance of the electrode was 1300 megohms for a length of 1·0 cm. The types of buffers employed and other pertinent information are indicated in the legend. The slope is close to 58 mv per pH unit which is the theoretical value for 21°.

When a given electrode did not meet the stringent requirements outlined above, occasionally but not very often its behaviour could be improved by such procedures as reinsulation, by aging in 0·1 N-HCl, by 'cycling' in acid and base, or finally by regeneration in hydrofluoric acid.

Insertion of electrodes. The giant axon was mounted horizontally across a glass plate 35–40 mm in width. The axon was longer than the width of the glass by several mm. The ends of the axon were tied with a fine thread, stretched and fixed to a platform that was located at a lower plane than the glass. This resulted in the axon being bent downward at the edge of the glass plate. With a pair of fine scissors, two small incisions were made on the uppermost surface of the axon, each in a region of the axon near the edge of the plate. With the aid of a micromanipulator, the glass electrode was inserted through one of these holes into the axon and advanced along the long axis of the axon (Fig. 3, left). With the aid of a second micromanipulator, the glass capillary portion of the micro-calomel

electrode was inserted longitudinally into the axon through the second hole (Fig. 3, right). By careful manipulation of the micromanipulators, the two inserted electrodes were arranged in the axon in such a manner that the tip of one met the tip of the other, or so that some overlapping was achieved.

Fig. 3, is a photomicrograph of the glass electrode and micro-calomel electrode in position in the axon. The electrodes have been so positioned that their tips have met. The electrode to the left is the glass electrode (its tip is slightly enlarged by fusing). The electrode to the right is the calomel electrode. During excision, the part of the stellar nerve and other connective tissue to one side of the axon was dissected away. The stellar nerve is still seen attached to the other side of the giant axon. The agar–sea water portion of the macro-calomel electrode was, as a rule, immersed in the sea water bathing the preparation.

In a series of experiments, an attempt was made to measure the pH of the interstitial space. In these experiments, the giant axon was excised together with the stellar and fin nerves. This compound nerve was mounted on the glass plate and the glass and micro-calomel electrodes were inserted in much the same way as was used for the giant axon alone. Care was taken that damage to nerve fibres was minimized during insertion.

Electric instruments. The potential difference between the glass and the calomel (macro- or micro-) electrodes was measured by means of a Kintel DC micro-voltmeter. The circuitry is illustrated in Fig. 1. Previous to the measurement of the potential difference between the glass electrode and either of the calomel electrodes, a determination was made of the resting membrane potential by measuring the potential difference between the macro-calomel electrode, immersed in the fluid bathing the axon, and the micro-calomel electrode inserted into the axon. The excitability of the axon was tested by recording the action potential. This was accomplished by measuring the potential difference between the large volume of sea water surrounding the fibre and the interior of the axon with the macro-calomel and micro-calomel electrodes. The calomel electrodes were led to a cathode follower which utilized a Z729 Emitron tube in triode connection. The response was displayed upon the screen of a Dumont 322 dual beam oscilloscope and photographed with a Grass Kymograph Camera. The axon was stimulated by means of electrical pulses applied through a pair of platinum wires making contact with one end of the axon. The electrical pulses were obtained from a Grass S4A Stimulator and were isolated from ground by means of a Grass S1U4A isolation unit.

Prior to the measurement of the potential difference between the glass and the calomel electrodes, the connections to the cathode follower and the stimulator were removed. If measurements are reliable, the potential difference between the glass and the micro-calomel electrodes, plus the potential difference between the micro-calomel and the macro-calomel electrodes, should equal the potential difference between the glass and the macro-calomel electrodes. The sequence of measuring the potential difference between glass and micro-calomel, glass and macro-calomel, and macro-calomel and micro-calomel electrodes was repeated for a given position in the axon several times. Furthermore, this procedure was repeated when the micro-calomel or the glass electrode or both were moved along the axon to different positions. The limit of accuracy of measurement of the potential difference between the glass electrode and the calomel electrode was usually 1 mv. With some electrodes, however, the accuracy was not greater than 1·5 mv. The error in measurement of the potential difference between the two types of calomel electrodes was less than 0·1 mv.

Method for extrusion and titration of axoplasm. Primarily for the purpose of determining the buffering capacity of axoplasm, an attempt was made to carry out a potentiometric titration. The axoplasm was extruded as follows: after excision of the axon, as much as possible of the adhering sea water on the surface was removed by resting the axon on a piece of clean absorbing paper. The axon, with one end cut open, was mounted on a clean, dry glass plate. The cut end was under observation with darkfield illumination. The axoplasm was squeezed out of the cut end by pressing a glass rod against the axon near the uncut end. This end of the axon was held motionless and the glass rod, while still pressing against the axon and the underlying glass plate, was advanced towards the cut end of the axon. Frequently extrusion was aided by pulling the axon near the cut end with the tip of a needle. This general procedure assured minimal contamination of axoplasm with sea water or other tissue material.

Two methods were used for titrating the axoplasm. At first a very small amount of axoplasm, approximately 0·005 ml, was sucked up in a very fine glass tube, approximately 0·5 mm internal diameter. In this tube were inserted the glass and the micro-calomel electrodes much in the same manner in which they were inserted in the axon. After each addition of acid or base, the pH was

VOL.
5
1959/6

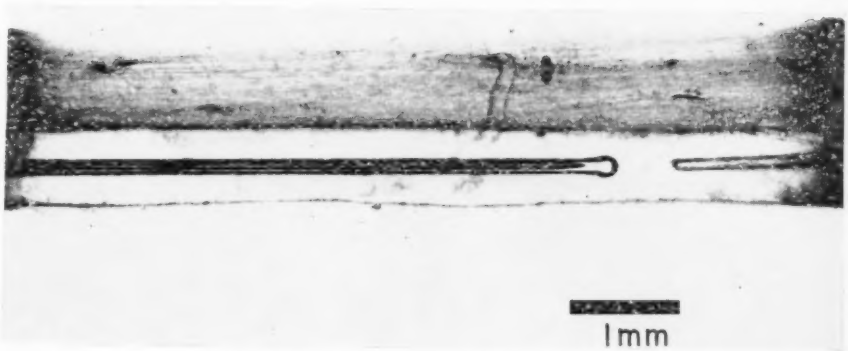


FIG. 3.—Photomicrograph of giant axon with inserted microglass pH electrode (left) and calomel electrode (right).

VOL.
5
1959/6

determined. In later experiments, when it was known that the buffering capacity of axoplasm was very high, titrations were carried out by diluting axoplasm obtained from 30–35 axons, by 50–100 times. This gave a volume of fluid of 1–2 ml. This volume was sufficient to carry out a titration with a standard G model Beckman pH meter.

RESULTS

pH *value of the axoplasm.* In establishing that the measurement of pH was reliable, not only was it necessary to check the electrodes but also to make certain that the membrane potential was uniform along the length of axon where the glass electrode was located. The potential difference across the membrane, measured with the micro-calomel and the macro-calomel electrodes, was 55–61 mv, the inside of the membrane being negative to the outside. Over the length of axon where the glass electrode was located, the variation was usually less than 1 mv.

The potential difference obtained between the glass and the micro-calomel electrodes corresponded to a pH in the range of 7·3–7·5. As the glass electrode was kept stationary and the microcalomel electrode moved along the long axis of the axon, the potential difference did not vary by any measurable amount (note: 1·5 mv is the limit of accuracy of measurement). In the few instances in which it did vary by more than 2 mv, the data were discarded. In a similar manner, by keeping the micro-calomel electrode stationary and moving the glass electrode along the axon, the changes in the potential difference between the electrodes were usually less than 2 mv. When the change was larger than 2 mv, the data were discarded.

As mentioned earlier, the potential difference between the glass and the micro-calomel electrodes plus the potential difference between the micro-calomel and the macro-calomel electrodes should equal the potential difference between the glass and the macro-calomel electrodes. This was found to be true, usually within 1·5 mv. The potential difference across the glass and the macro-calomel electrodes usually varied by less than 2 mv when moving either the glass or the micro-calomel electrode. Out of the 65 fibres employed, none was found that showed a potential difference between the glass and the calomel electrodes that corresponded to a pH outside the range of 7·3–7·5. To some extent at least, this range may be a reflection of the accuracy of our technique rather than an inherent variation from fibre to fibre. A change in potential difference of approximately 5·8 mv corresponds to a change in pH of 0·1 units. Considering inherent variation in the resting potential, the effects upon the potential measurement of moving the internal electrodes, the inaccuracy of measuring the potential difference across the glass electrode, it would seem that there exists a maximal uncertainty in our measurement of pH of the order of magnitude of 0·05–0·1 units.

This pH range of 7·3–7·5 should probably be decreased by about 0·05 to make allowance for liquid junction potentials. This value was obtained by CALDWELL (personal communication), who, by using PLANCK's or HENDERSON's equation, has calculated the liquid junction potential for a variety of electrode systems. He has verified experimentally the values obtained theoretically. It may be stated then that, according to these experiments, the axpolasmic pH is 7·35 ($\pm$0·1).

In two experiments, the pH of the axoplasm was measured after the fibre was excised without bringing it into contact with sea water. The measurements were made with the surrounding stellar nerve intact. The pH obtained was similar when the axon was immersed in sea water. Fresh sea water had a pH of approximately 7·95.

When exposed to air for prolonged periods of time the pH may drop by 0·1 units or more.

The effects upon the axoplasmic pH *of a number of agents.* Since, as mentioned above, the error in measuring axoplasmic pH was not smaller than 0·05–0·1 units, an agent was considered to 'affect' axoplasmic pH only if the change found was greater than this. A number of agents were found to be without a detectable effect. These included replacing the sea water with isotonic choline choride, isotonic sucrose, sea water containing 5% ethanol, and sea water containing 3% urethane. These agents are known to make the axon reversibly 'inexcitable'. They were permitted to be in contact with the fibre for approximately 1–2 min prior to pH determinations. This period of time is sufficient to render a cleaned axon 'inexcitable'.

A number of agents were found to affect the internal pH. The axoplasmic pH decreased by 0·1–0·4 units after exposing the axon to saturated KCl, NaCl, hot sea water, chloroform, ethanol and ethylene dichloride and after crushing the axon. These are agents producing irreversible injury to the axon. Determinations of pH were made not during but after the injurious agent was removed and the axon was returned to normal sea water. The agent was permitted to be in contact with the axon for approximately 0·5 to 1 min, a period of time more than sufficient to make the axon irreversibly inexcitable. A 10° drop in temperature was accompanied by a drop in potential of 3–9 mv. The effect of temperature was reversible. The effect of temperature was not very pronounced. Since the effects were close to the error of measurement by the present method, the values given above should be taken only as an indication of the order of magnitude of the effect.

The effects of changing the external pH were not always reproducible. In addition, different types of buffers and the same type of buffers of different strengths produced different effects. Nevertheless it appeared clear that for short periods of time the axoplasmic pH showed considerable independence of the external pH. The following are examples of this type of experiment. Sea water was acidified to pH 6·0 by adding 0·33 g of $NaH_2PO_4 \cdot H_2O$ in 900 ml of sea water. When the axon was bathed in such a solution, within five minutes the internal pH decreased by 0·1–0·15 units. When sea water was acidified to pH 5·6 by the use of 0·1 N-HCl, within five minutes the internal pH dropped by 0·1–0·2 units. This problem will not be treated in any greater detail. CALDWELL has studied it quite thoroughly and the present result appears to be in agreement with his findings (cf. Discussion).

pH *of interstitial space.* An attempt was made to determine the pH of the interstitial space of the stellar nerve of *Loligo pealii*. The reason for this undertaking was to obtain some information about the pH of the various environments to which the axon is exposed *in vivo*. When the glass and the micro-calomel electrodes were inserted into the interstitial space in an excised compound nerve composed of the stellar and fin nerves (see Methods), a potential difference was recorded between the two electrodes which corresponded to a pH of 7·0–7·1. The result was consistent in four different preparations. There is, however, some doubt as to the reliability of this measurement (cf. Discussion).

Potentiometric titration of axoplasm. As mentioned in the section on Methods, two potentiometric methods were employed for determining the titration curve of axoplasm. The results obtained by both methods were the same. The pH of extruded axoplasm was in all instances 6·9–7·1. This was so whether it was measured undiluted

VOL. 5 1959/6

or diluted 5-fold or 100-fold with isotonic KCl or distilled water. When diluting the axoplasm with distilled water or with isotonic KCl, a small amount of axoplasmic material did not go into solution.

The volumes of axoplasm used in obtaining the titration curve (Fig. 4) were not identical, but the divergence of the curve due to this small difference in the volume is considered to be negligible. The reason for using different samples of axoplasm for titration in the acid and alkaline ranges was the finding that, following titration with the base, the configuration of the curve was somewhat altered. In this experiment (Fig. 4) a Model G Beckman pH meter was employed.

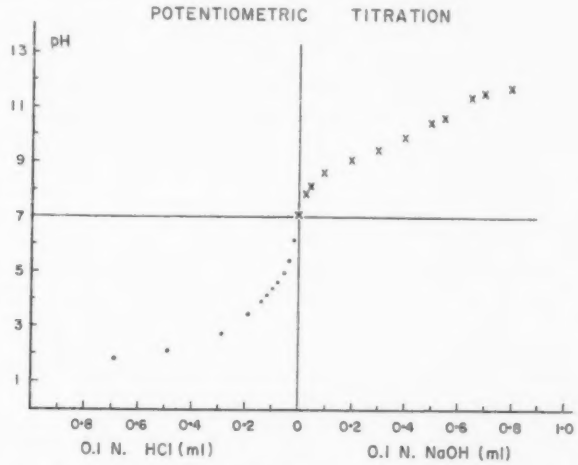


FIG. 4.—Titration curve of axoplasm. Ordinates indicate pH. Abscissae indicate amount of 0·1 N-HCl or 0·1 N-NaOH added. This is a composite curve. When titration started with acid (dots, left), the volume of axoplasm was 0·11 ml.. To this was added 0·9 ml of isotonic KCl. The pH of the mixture was 7·1. When titration started with base (crosses right), the volume of the axoplasm was 0·12 ml. To this was added 0·9 ml of KCl. The pH of the mixture was 7·05. The Beckman Model G pH meter was employed. The temperature was 21°C.

In the curve there is a plateau at pH 9–10 which may be indicative of a strong buffering capacity (see Discussion).

The effects of extracellular pH *upon the electrophysiological properties of the axon.* Making the external sea water acid or alkaline affects the amplitude of the action potential and the size of the resting potential. Acidifying sea water results in a reduction in the magnitude of the resting potential and of the amplitude of the action potential. The results obtained by making sea water more alkaline are the opposite. These results were not clearly reproducible from axon to axon; furthermore, they seemed to depend upon the strength and type of buffer employed.

Examples of these experiments are the following: When the pH of sea water bathing the axon was adjusted to 5·5 by titration with 0·1 N-HCl, the amplitude of the action potential decreased by 7–9 per cent and the resting potential by 6–10 per cent. When sea water was acidified to pH 4·1 with acetate buffer (5×10^{-3} M), the action potential decreased by 60 per cent and the resting potential by 30–33 per cent. When sea water was acidified to pH 5·5 with 10^{-3} M-acetate buffer, the amplitude of the action potential decreased by 10 per cent and the resting potential by approximately 15 per cent. When sea water was acidified with NaH_2PO_4 ($2{\cdot}4 \times 10^{-3}$ M) to pH 5·6,

the amplitude of the action potential decreased by about 4–5 per cent and the magnitude of the resting potential by 6–11 per cent.

DISCUSSION

ARVANITAKI and CHALAZONITIS (1951; 1954) injected bromo-cresol purple into the sepia giant axon and obtained a colour which corresponded to a pH of 6·6. They reported no spread of pH values in their axons. CHAMBERS and KAO (1952) injected phenol and bromo-cresol purple into the giant axon of *Loligo pealii* and obtained a colour which corresponded to a pH of 6·8–7·0. They did not report a different pH in different axons. CALDWELL (1958), using a glass electrode, found a different pH for different axons, ranging between 6·7 and 7·3. He implied that this scatter was not inherent in his method.

The discrepancies between the present experimental work and the previous work on nerve fibres are somewhat minor and reside (1) in whether or not the pH is as high as 7·35 (±0·1) and (2) in whether or not the variation of pH among different axons is very small. On a number of other issues, the present results are in agreement with CALDWELL's.

Some reassurance on the validity of the present technique of measuring cytoplasmic pH was obtained through the work on extruded axoplasm. When the axoplasm is extruded, it is in a gel form. By using a procedure identical to that employed with intact axons, the pH of the extruded axoplasm was found to be about 7·0. When the extruded axoplasm was diluted 5- to 100-fold with distilled water or isotonic KCl, the gel and other structures were disrupted, and with the same technique, the pH value of 7 or thereabout did not change by more than 0·1 units. At this point the volume was large enough to make a determination using the large glass electrodes, as in a standard pH determination on a fluid with no structure and with extremely low concentration of protein. There is little doubt as to the reliability of this pH measurement. This finding suggests that the reading with the small glass electrode in extruded undiluted axoplasm also was reliable.

In the Results it was mentioned that any form of severe injury of the axon (e.g. with ethanol, ethylene dichloride, chloroform, hot sea water, saturated salt, or crushing) resulted in a pH which was often near 7·0–7·1. It seems possible that the reason for the pH of expressed axoplasm being as low as 7·0 was the injury incident to its extrusion. This conjecture, stated differently, would mean that not only was the measurement (with the small glass electrode) of the pH of extruded axoplasm reliable, but also the measurement of the pH inside the impaired or even the normal axon. The possibility naturally exists that *in vivo* the cytoplasmic pH may be different.

Since there was no appreciable difference in pH between the extruded axoplasm disrupted by dilution and the gelated extruded axoplasm, it would seem that close contact of the glass electrode with cytoplasmic inclusions such as particulates and vacuoles did not markedly affect the reading. Pertinent in this connection is the work of DANIELLI (1951) indicating that a glass electrode is sensitive to the hydrogen ion concentration only of the bulk phase of protein solutions.

The possibility exists that a film or membrane may be interposed between the glass electrode and the axoplasm. Certain cells are known to produce such films in the presence of foreign objects. Since there was no appreciable difference in pH between the undiluted and diluted axoplasm, the possibility that such an encapsulating film, if

VOL.
5
1959/6

present, markedly affects my reading is unlikely. This would be so unless injury to the cell abolished the intervention of such an envelope in the measurement of axoplasmic pH.

SPIEGLER and WYLLIE (1956), and others, have emphasized the point that, when attempts are made to measure the pH of a paste, gel, or thick suspension with the potentiometric method, the potential recorded may not merely reflect the hydrogen ion concentration. They point out that the insertion of the salt bridge into the suspension may be responsible for an additional potential. In some instances, they report this potential may give rise to an error in pH of the order of 2 units. In my procedure, there seems to be little need for concern over this source of error as my reading of pH for the axoplasm was not appreciably affected after the gelated extruded axoplasm was disrupted by extreme dilution.

In CALDWELL's experiments, most of the axons employed had very low resting potentials. The range of resting potentials in the present study was 55–61, with most axons having a value very close to 60. Most likely a large part of the variation in pH values which CALDWELL observed (6·7–7·3) may be due to partial injury of the axons used. In injured axons the membrane potential is usually not uniform. As mentioned earlier, uniformity of membrane potential is required for reliable pH measurements. If one were to disregard CALDWELL's results obtained with axons with low resting potentials, his results would be in closer agreement with the present ones. There are in CALDWELL's method several additional minor shortcomings. These relate to the unorthodox use of copper wire in the glass electrode, the use of a reference electrode other than calomel, the setting of a limit of slopes for usable glass electrodes as low as 45 mv per pH unit, and the fact that the excitability of the axons was not tested.

One of the main reasons why CALDWELL as well as the present author undertook to study the internal pH was to ascertain whether or not the distribution of hydrogen ions across the membrane is a Donnan distribution. If the extracellular and intracellular hydrogen ion activities were governed by a Donnan equilibrium, then the membrane potential in mv should be equal to 58 × [intracellular pH—extracellular pH]. CALDWELL concluded that this was not so. The present author is in agreement, with the reservation that this conclusion applies only to the pH of the bulk of axoplasm (axoplasm in contact with glass electrode) and only to the pH of the bulk of extracellular fluids. The present method does not give any information as to the pH barely under the surface of the cell or just barely outside the surface membrane. It is possible that a number of agents which do not affect axoplasmic pH affect the pH barely under the membrane. Similarly, the agents affecting 'axoplasmic pH' may affect the pH of the axoplasm in the vicinity of the membrane differently.

ARVANITAKI and CHALAZONITIS (1954) and later CALDWELL (1958) reported that axoplasmic pH was relatively unaffected by changes in the pH of the extracellular medium unless such changes were brought about by CO_2 enrichment. In the present study, the effects of CO_2 were not studied.

It was stated under Results that irreversible processes are associated with titration. The irreversibility may mean that the titration curve (a) in large part does not express the buffering capacity of axoplasm or (b) that it still expresses the buffering capacity but that the material responsible for this capacity is affected by extreme pH.

If the latter possibility is correct, the titration curve indicates that basic groups must be an appreciable constituent of axoplasm. This information is not available from

the literature to date. Similarly, if the latter view is correct, the principal buffer is not a carbonate, phosphate, or amino acid because the titration curve should show two regions of strong buffering capacity and not one. We have no explanation of the existence of maximal buffering capacity in the region of pH other than the normal.

In the Results it was mentioned that an increase in extracellular pH increases the resting potential and a decrease decreases the resting potential. Preliminary experiments in this laboratory with injection of acid or base into the axon indicate that the resting potential is even more sensitive to changes in internal pH. It is interesting to note that after raising the concentration of potassium outside to 550 mM (and thus bringing the resting potential close to zero) one can restore a 'membrane' potential of either sign by adjusting the inside and outside pH.

Finally, a brief comment will be made concerning the finding that when the glass electrode was inserted into the interstitial space a potential was registered corresponding to a pH of about 7. It must be emphasized that there is little assurance that the potential recording of the glass when in intimate contact with the surfaces of nerve fibres necessarily gives an indication of the pH of interstitial fluid. The pH of the squid blood is known to be 7·4. It is interesting that the pH of axoplasm is very close to that of the blood.

VOL.
5
1959/6

SUMMARY

A glass pH electrode and a calomel electrode were inserted into the giant axon of *Loligo pealii*. The potential obtained when the axon was bathed in sea water corresponded to a pH of 7·35 (±0·1). A number of agents were found which did, and a number which did not, affect the axoplasmic pH. A potentiometric titration of extruded axoplasm was carried out. This indicated that at normal pH the buffering capacity of axoplasm is very high and that the maximal buffering capacity lies in the pH range of 9–10. The pH of the bathing medium was found to affect the electrophysiological properties of the axon.

Acknowledgement—The author wishes to acknowledge with gratitude the help of Dr. ICHIJI TASAKI.

REFERENCES

ARVANITAKI A. and CHALAZONITIS N. (1951) *Arch. Sci. physiol.* **5,** 207.
ARVANITAKI A. and CHALAZONITIS N. (1954) *C. R. Soc. Biol., Paris* 1948, 952.
CALDWELL P. C. (1956) *Internat. Rev. Cytol.* **5,** 229.
CALDWELL P. C. (1958) *J. Physiol.* **142,** 22.
CHAMBERS R. (1949) *Biol. Rev.* **24,** 246.
CHAMBERS R. and KAO C. (1952) *Exp. Cell Res.* **3,** 564.
CLARK W. M. and LUBS H. A. (1916) *J. biol. Chem.* **25,** 479.
DANIELLI J. F. (1951)
GRÄFF S. and RAPPOPORT E. (1937) *Ergebn. allg. Path. path. Anat.* **33,** 181.
HEILBRUNN L. V. (1952) *An Outline of General Physiology* (3rd Ed.) Saunders, Philadelphia.
LISON L. (1941) *Tabul. biol., Amst.* **19,** 1.
OSTROW J. D. and RUSSELL J. B. (1951) *Marine Biological Laboratory Formulae and Methods* Woods Hole, Mass.
REISS P. (1926) *Le* pH *Interieur Cellulaire.* Paris.
SMALL J. (1955) *Protoplasmatologia, Wien* **2,** B2c.
SORENSON S. P. L. (1912) *Ergebn. Physiol.* **12,** 393.
SPIEGLER K. S. and WYLLIE M. R. J. (1956) In *Physical Techniques in Biological Research* (Edited by OSTER G. and POLLISTER A. W.) Vol. 2, p. 301. Academic Press, New York.
TASAKI I. (1953) *Nervous Transmission.* Thomas, Springfield, Ill.
WALPOLE G. S. (1914) *J. Chem. Soc.* **105,** 2501.
WIERCINSKI F. J. (1955) *Protoplasmatologia, Wien* **2,** B2c.

Journal of Neurochemistry, 1960, Vol. 5, pp. 195–205. Pergamon Press Ltd. Printed in Northern Ireland

PRELIMINARY NOTES

Developpement de la cholinesterase au niveau du mesencephale chez l'embryon de poulet

Development of cholinesterase in the mesencephalon of the chick embryo

(*Received* 20 *July* 1959)

OL.
5
9/60

Le taux d'activité de la cholinestérase augmente dans les centres nerveux au cours de la vie embryonnaire (Nachmansohn, 1939; Kavaler et Kimel, 1952; Boell, Greenfield et Shen, 1955). Les travaux de divers auteurs tendent à démontrer qu'il existe un certain parallélisme entre la différenciation biochimique et l'apparition de la spécificité fonctionelle (Flexner, 1955; Sawyer, 1955). Ainsi, chez la larve d'Amblystoma, il existerait une relation quantitative entre la capacité réflexe à un stade donné du développement et le taux de cholinestérase spécifique à l'intérieur du complexe neuromusculaire (Sawyer, 1955). L'augmentation du taux d'activité de la cholinestérase pourrait alors être un critère assez convenable d'une certaine activité bioélectrique (Nachmanson and Wilson, 1956), donc d'une activité fonctionnelle spécifique en ce qui concerne les centres nerveux.

Dans un travail antérieur, nous avions poursuivi une étude histochimique de l'évolution et de la localisation de la cholinestérase au cours de la différenciation des diverses strates cellulaires du tectum des lobes optiques chez l'embryon de Poulet (Bonichon, 1957*b*). Mais les limites de l'histochimie microscopique sont assez étroites, car d'une part, les résultats obtenus ne sont pas quantitatifs, et d'autre part de fausses localisations, dues à une certaine diffusion des réactifs et de l'enzyme, peuvent toujours être inconnues (Lumsden, 1957). Seuls nous semblent avoir une certaine valeur, en tant qu'indice d'activité fonctionnelle spécifique, les résultats quantitatifs fournis par la chimie tissulaire.

Dans le présent travail nous avons effectué des dosages répétés d'activité de la cholinestérase, au niveau du mésencéphale, au cours de la majeure partie de la vie embryonnaire du Poulet (race Rhode Island Red). Les premières mesures ont été effectuées au stade 29 du développement (ces stades étant déterminés selon les critères morphologiques définis par Hamilton (1952)), soit au 6 ème jour d'incubation environ, et elles se poursuivent jusqu'à l'éclosion. Les stades antérieurs au stade 29 n'ont pas été étudiés, le mésencéphale étant alors trop peu développé pour permettre un isolement tissulaire convenable. L'activité cholinestérasique a été évaluée, quantitativement, par la mesure de la vitesse d'hydrolyse d'une solution de chlorhydrate d'acétylcholine, en utilisant la méthode manométrique classique de Warburg (Umbreit, Burris et Stauffer, 1957). Le tissu a été prélevé par dissection, selon la méthode et à l'aide de l'appareillage préconisés par Gayet (1955), dans du liquide physiologique (solution de NaCl à 9 g/l), puis il a été homogénéisé au moyen d'un appareil de Potter—Elvehjem muni d'un piston en Teflon. Les homogénats ont été préparés dans la solution physiologique de Ringer, tamponnée au bicarbonate à pH 7·4, selon le procédé d'Augustinsson (1957) puis dilués au I/50 ème. L'hydrolyse enzymatique a eu lieu en atmosphère constituée par le mélange de 5% CO_2 et 95% N_2, à la température de +37°. Pour chacune de nos expériences nous avons utilisé une fiole témoin, dans laquelle la préparation enzymatique a été supprimée et remplacée par le même volume de solution physiologique. La durée de l'expérience n' a jamais excédé 30 min, et, aux stades correspondant à l'éclosion ou les précédant directement (stades 43 à 46) elle a été réduite à 10 min. Le volume de CO_2 dégagé est rapporté d'une part au poids frais, et d'autre part à l'azote total tissulaire. Pour assurer ce dernier dosage le tissu est minéralisé grace au mélange de Needham et Boell (1939) au bain de sable électrique. Les dosages d'azote total sont effectués selon la méthode de microdiffusion de Conway (1957)(titrage à l'aide d'une micro-seringue automatique Agla et d'un micro-agitateur électromagnétique). L'activité enzymatique est exprimée en μl CO_2/μg N_2 tissulaire heure. Le Tableau 1 contient l'ensemble de nos résultats. L'évolution de l'activité enzymatique est représentée par le Figure 1.

Au stade 29 l'activité enzymatique est déjà manifeste: l'hydrolyse est faible et les différentes mesures effectuées donnent des résultats assez inconstants. Aux stades suivants les résultats sont plus réguliers, l'activité cholinestérasique croît très légèrement d'un stade à l'autre, et de façon régulière jusqu' au stade 35 où elle atteint sensiblement une valeur double de celle que nous avions obtenue au stade 29.

Du stade 35 au stade 40 le taux d'activité enzymatique passe de deux à trois fois sa valeur initiale Mais cette augmentation n'est pas progressive: on peut y distinguer une première phase correspondant aux stades 35, 36 et 37, durant laquelle le taux d'activite augmente rapidement, suivie d'une seconde phase correspondant aux stades 37, 38 et 39 et caractérisée par une certaine stabilisation de l'activité

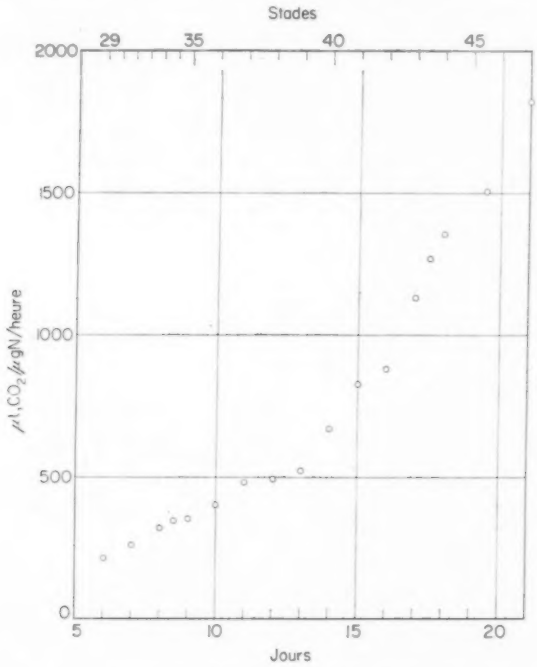


FIG. 1—Développement de l'activité de la cholinestérase au niveau du mésencéphale de l'embryon de Poulet

cholinestérasique. Le Tableau 1 indique le nombre d'expériences faites au cours de ces deux phases critiques, et qui nous permettent d'interpréter nos résultats dans ce veno.

A partir du stade 40 l'hydrolyse enzymatique croîe très rapidement, pour atteindre sens fois sa valeur initiale au stade 46, soit a l'éclosion.

L'activité de la cholinestérase, bien que très faible, apparait donc très précocement dans le mésencéphale embryonnaire, alors que la différenciation morphologique est à peine ébauchée (le nucléole apparait au stade 29 dans les noyaux des cellules de la strate la plus externe du tectum (BONICHON 1957*a*) et qu' aucune activité fonctionnelle n'est décélable (PETERS, VONDERAHE et POWERS, 1956). L'augmentation du taux d'activité de la cholinestérase, qui débute réellement aux stades 35–36, coïncide avec l'acquisition par les cellules mésencéphaliques des caractères structuraux (nucléole presque dépourvu de matériel Feulgen-positif et entouré du 'nucleolus associated chromatin'; volume nucléaire maximum) et cytochimiques (augmentation de l'acide ribonucléique cellulaire localisé surtout au niveau des corps de Nissl; augmentation d'activité de la phosphatase alcaline) (BONICHON, 1957*a*) qui font du neuroblaste un neurone typique. Simultanément, la consommation d'oxygène qui s'était maintenue à un taux constant augmente brusquement et ne cesse de croître jusqu'à l'éclosion (GAYET, 1955). Les premiers signes d'activité bioélectrique du mésencéphale ont été décélés au 10 ème

BIOL.
9/60

TABLEAU 1.—DÉVELOPPEMENT DE L'ACTIVITÉ DE LA CHOLINESTÉRASE AU NIVEAU DU MÉSENCÉPHALE DE L'EMBRYON DE POULET EN FONCTION DU STADE MORPHOLOGIQUE ET DE LA DURÉE D'INCUBATION

Stades	28–29	31	33	34	35	36	37	38	39	40	41	42	43	43–44	44	Avant éclosion	Eclosion	Après éclosion
Jours	6	7	7–8	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
μl CO_2 dégagé par fiole* et par heure	100 ±21·6	110 ±34·5	142 ±19·7	147 ±21·5	151·2 ±35·3	174 ± 13·6	206·5 ±19	207·5 ±14·9	226·3 ±18·5	275·3 ±25·4	329 ±20·2	357·7 ±25·3	440	525	616 ±30·2	692 ±47·7	824 ±47·6	893 ±67·7
μl CO_2 dégagé par μg Azote tissulaire et par heure	217·5 ±42·8	263·9 ±50·1	324 ±26·4	350 ±14·2	355·5 ±14·1	404·7 ±47·4	488·9 ±44·2	499 ±35·4	525 ±43·7	674 ±23·5	828·9 ±40·9	881·3 ±58·2	1131·3	1273	1352 ±141·6	1507 ±45·2	1821 ±31·3	1775 ±116
Nombre d'expériences	5	7	8	4	12	11	11	16	11	12	11	12	3	3	12	6	6	9

* Volume de CO_2 dégagé correspondant à 1·6 ml d'homogénat tissulaire au 1/50 ème auquel on ajoute 0·4 ml de la solution de chlorhydrate d'acétylcholine de concentration $11{\cdot}05 \times 10^{-3}$ M.

jour de développement embryonnaire, c'est à dire au stade 36 (PETERS, VONDERAHE et POWERS, 1956), au moment donc où l'activité cholinestérasique s'accélère.

Le développement de l'activité de la cholinestérase s'intégrerait ainsi dans le schéma évolutif du métabolisme général du tissu mésencéphalique embryonnaire. La différenciation biochimique suivrait un cours sensiblement parallèle à celui de la différenciation fonctionelle, bien que l'apparition d'une activité bioélectrique (rythme spontané) ne soit pas accompagnée d'un changement spectaculaire du taux d'activité enzymatique. Peut-être faut-il que la concentration en cholinestérase atteigne un certain seuil pour que l'activité bioélectrique se manifeste spontanément: on a pu constater qu'il fallait qu'une concentration déterminée d'acétylcholine soit atteinte pour que le coeur se mette à battre rythmiquement (FELDBERG, 1957). Des substances, comme le Métrazol, qui permettent d'induire une activité électrique précoce au niveau de l'encéphale de l'embryon de Poulet (PETERS, VONDERAHE et POWERS, 1956) pourraient éventuellement intervenir en abaissant ce seuil.

SUMMARY

A manometric analysis has been made of the development of cholinesterase in the mesencephalon of the chick embryo. The appearance and subsequent development of the enzymic activity parallel the general pattern of maturation of the embryonic nervous tissue as revealed by previous biochemical, histological and histochemical studies.

Remerciements.—L'auteur tient à exprimer ses remerciements au Prof. J. GAYET pour son aide constante au cours de ce travail ainsi que pour la préparation du manuscrit.

A. BONICHON

Laboratoire de Physiologie Générale
Faculté des Sciences
Université de Nancy, France

BIBLIOGRAPHIE

AUGUSTINSSON K. B. (1957) *Methods of Biochemical Analysis* (Edited by GLICK D.) Vol. 5 Interscience, New York.

BOELL E. J., GREENFIELD P. and SHEN S. C. (1955) *J. exptl. Zool.* **129,** 415.

BONICHON A. (1957*a*) *Bull. biol. France et Belg.* **XC°,** 158.

BONICHON A. (1957*b*) *C. R. Acad. Sci.* **245,** 1345.

CONWAY E. J. (1957) *Microdiffusion Analysis and Volumetric Error*. Crosby and Lockwood, London.

FELDBERG W. (1957) *Metabolism of the Nervous System* (Edited by RICHTER D.). Pergamon Press, London.

FLEXNER L. B. (1955). *Ann. N.Y. Acad. Sci.* **60,** 986.

GAYET J. (1955) *Arch. Sc. Physiol.* **9,** 11.

HAMILTON H. L. (1952) *Lillie's Development of the Chick*. Willier, New York.

KAVALER F. and KIMEL V. M. (1952) *J. comp. Neurol.* **96,** 113.

LUMSDEN C. E. (1957) *Metabolism of the Nervous System* (Edited by RICHTER D.). Pergamon Press, London.

NACHMANSOHN D. (1939) *Bull. Soc. Chim. Biol.* **21,** 761.

NACHMANSOHN D. and WILSON I. B. (1956) *Currents in Biochemical Research* (Edited by GREEN D. E.) Interscience, New York.

NEEDHAM J. and BOELL E. J. (1939) *Biochem. J.* **33,** 149.

PETERS J. J., VONDERAHE A. R. and POWERS T. H. (1956) *J. exptl. Zool.* **133,** 505.

SAWYER C. H. (1955) *J. exptl. Zool.* **129,** 561.

UMBREIT W. W., BURRIS R. H. and STAUFFER J. F. (1957) *Manometric Techniques.* Burgess Minneapolis.

VOL.
5
1959/6

Potassium accumulation in guinea pig brain cortex slices

(*Received* 20 *August* 1959)

SCHATZMAN (1953) found that different cardiac glycosides inhibit potassium accumulation in red blood cells, and similar results were obtained in other tissues (MATCHETT and JOHNSON, 1954; WOLLENBERGER and WAHLER, 1956; VICK and KAHN, 1957; WHITNEY and WIDDAS, 1959). In previous experiments (GÁRDOS, 1958*a*, *b*; 1959) it was shown that ethylenediamine tetraacetic acid (EDTA) also inhibits potassium transport in human erythrocytes by chelating the calcium, which is concerned in the mechanism of transport. In the present experiments the effect of these agents on the transport of potassium ions in brain cortex slices was investigated.

KREBS and co-workers (TERNER, EGGLESTON and KREBS, 1950; KREBS, EGGLESTON and TERNER, 1951) demonstrated that guinea pig brain cortex slices maintain their optimum potassium level when incubated in the presence of glucose and L-glutamate under aerobic conditions. In our experiments guinea pig brain cortex slices (100 mg wet weight per vessel) were incubated in 2 ml of a standard medium containing 0·136 M-NaCl, 0·006 M-KCl, 0·03 M-tris-HCl buffer (pH 7·4) (GÖMÖRI, 1955), 0·02

TABLE 1.—EFFECTS OF VARIOUS AGENTS ON THE POTASSIUM CONTENT OF BRAIN CORTEX SLICES

Expt. No.	Agents added	μ moles K^+/g tissue		
		Initially	After incubation	Change
1.	None	73·0	21·0	−52·0
	Glucose, L-glutamate	73·0	56·0	−17·0
	Glucose, L-glutamate, 10^{-4} M-EDTA	73·0	51·5	−21·5
	Glucose, L-glutamate, 10^{-3} M-EDTA	73·0	16·5	−56·5
	Glucose, L-glutamate, 10^{-3} M-EDTA, 10^{-2} M-$CaCl_2$	73·0	50·7	−22·3
	Glucose, L-glutamate, 10^{-3} M-EDTA, 5×10^{-3} M-$MgSO_4$	73·0	48·7	−24·3
2.	Glucose, L-glutamate	84·0	56·0	−28·0
	Glucose, L-glutamate, 5×10^{-6} M-ouabain	84·0	11·3	−72·7
	Glucose, L-glutamate, 5×10^{-6} M-ouabain, 5×10^{-3} M-$CaCl_2$	84·0	27·6	−56·4
	Glucose, L-glutamate, 5×10^{-6} M-oubain, 10^{-2} M-$MgSO_4$	84·0	23·6	−60·4

Aerobic incubation in Tris-saline for 60 min at 37°; 0·02 M-glucose, 0·01 M-L-glutamate. These data are from two animals.

M-glucose and 0·01 M-L-glutamate at 37° in air or in 100% O_2. The results were corrected for the swelling of the slices as in the experiments of TERNER, EGGLESTON and KREBS (1950).

The potassium accumulation obtained in the presence of glucose and L-glutamate was completely inhibited by 10^{-3} M-EDTA. When 10^{-3} M-$CaCl_2$ was added together with the EDTA the inhibition of the potassium accumulation by EDTA was prevented. Magnesium had a similar effect to calcium, but only at a higher concentration (Table 1). In control experiments the addition of 5×10^{-3} M-$CaCl_2$ or $MgSO_4$ to the standard medium increased the potassium content by 35 per cent. In the presence

of 5×10^{-6} M-ouabain (strophanthin-G) the potassium accumulation was also completely inhibited. The inhibition by ouabain was reduced in the presence of 5×10^{-3} M-$CaCl_2$ or 10^{-2} M-$MgSO_4$, but not completely prevented (Table 1).

In further studies of the kinetics of potassium transport, the slices were preincubated for 5 min at 37° in the above medium from which glucose and L-glutamate had been omitted. During this time the slices lost about 50 per cent of their potassium content. After preincubation, glucose, L-glutamate and various compounds were added to the medium. When EDTA was included in the system the slices could not regain their original potassium content; when $CaCl_2$ was also included the accumulation was the same as in the control (Fig. 1). The effect of ouabain was very similar to that of EDTA.

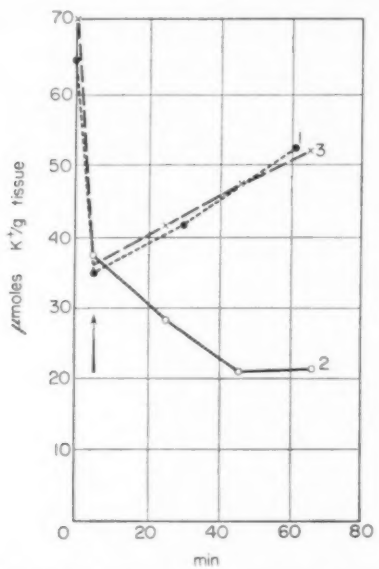


FIG. 1.—Kinetics of potassium transport in the presence of EDTA and $CaCl_2$. (1) Glucose and L-glutamate; (2) glucose, L-glutamate and 10^{-3} M-EDTA; (3) glucose, L-glutamate, 10^{-3} M-EDTA and 10^{-3} M-$CaCl_2$. The addition of substrates, EDTA and $CaCl_2$ was made after 5 min.

On the basis of these experiments it may be concluded that the calcium and magnesium of the cell membrane are important for potassium accumulation. In the case of EDTA the inhibitory effect on potassium accumulation could be directly attributed to the removal of the calcium and magnesium ions. It is possible that ouabain also acts through the removal of some of the bivalent cations of the membrane. As the effects of EDTA and ouabain in brain slices were very similar to those found in erythrocytes, muscle and frog skin, it is probable that potassium transport in different tissues is effected by a similar mechanism.

Acknowledgement—The author wishes to thank Dr. D. RICHTER for his advice and encouragement. The author also wishes to express his gratitude to the Welsh Regional Hospital Board for a personal grant.

G. GÁRDOS*

Medical Research Council Neuropsychiatric Research Unit
Whitchurch Hospital, Cardiff

REFERENCES

GÁRDOS G. (1958*a*) *Acta Physiol. hung.* **14,** 1.
GÁRDOS G. (1958*b*) *Biochim. biophys. acta* **30,** 653.
GÁRDOS G. (1959) *Acta Physiol. hung.* **15,** 121.

* Present address: Institute of Medical Chemistry, University of Budapest, Hungary.

VOL.
5
1959/6

GÖMÖRI G. (1955). In *Methods in Enzymology* (Edited by COLOWICK S. P. and KAPLAN N. O.) Vol. 1., p. 144. Academic Press, New York.
KREBS H. A., EGGLESTON L. V. and TERNER C. (1951) *Biochem. J.* **48,** 530.
MATCHETT P. A. and JOHNSON J. A. (1954) *Fed. Proc.* **13,** 384.
SCHATZMANN H. J. (1953) *Helv. Physiol. acta* **11,** 346.
TERNER C., EGGLESTON L. V. and KREBS H. A. (1950) *Biochem. J.* **47,** 139.
VICK R. L. and KAHN J. B. (1957) *J. Pharmacol.* **121,** 389.
WHITNEY B. P. and WIDDAS W. F. (1959) *J. Physiol.* **146,** 35 P.
WOLLENBERGER A. and WAHLER B. (1956) *Arch. exper. Path. u. Pharmakol.* **228,** 134.

OL.
5
9/60

Serotonin content of rat brain in relation to sex and age

(*Received* 21 *July* 1959)

DURING the last few years the progress in psychopharmacology has increased the interest in the significance of brain serotonin. During an extensive programme of research into a possible mechanism of action of psychotropic drugs, remarkable differences were observed in the brain serotonin of rats, according to the sex and age.

Sprague–Dawley rats were used in our experiments and killed by decapitation. The determinations of serotonin were carried out on brain using a Farrand spectrophotofluorimeter as described by BOGDANSKI *et al.* (1957). In some experiments, determinations were also carried out on hemisphere and brainstem.

The serotonin content of the brain (including cerebellum) of an adult male rat was 0·48 ± 0·014 μg/g while that of an adult female rat was 0·54 ± 0·013 μg/g. In foetuses and new born rats, the brain serotonin content was only 20–30 per cent of that of an adult male, but increased with age.

TABLE 1.—SEROTONIN CONTENT OF RAT BRAIN

Age	Sex	Brain weight g		Brain serotonin (μg/g ± S.E.)		
		Hemisphere	Brainstem	Whole brain	Hemisphere	Brainstem
Foetus*				0·10 ± 0·004 (4)		
2 days		0·24	0·09	0·17 ± 0·010 (4)		
5 days		0·36	0·16	0·21 ± 0·005 (4)		
10 days		0·67	0·29		0·17 (2)	0·46 (2)
15 days	M	0·76	0·33		0·18 (2)	0·43 (2)
25 days	M	0·88	0·39		0·22 (2)	0·65 (2)
35 days	F	0·92	0·45		0·27 (2)	0·69 (2)
45 days	M	0·98	0·47		0·40 ± 0·011 (3)	0·72 ± 0·025 (3)
	F	0·95	0·46		0·38 ± 0·013 (4)	0·74 ± 0·021 (4)
65 days	M	1·01	0·51		0·41 ± 0·033 (3)	0·74 ± 0·033 (3)
	F	0·94	0·50		0·41 ± 0·025 (3)	0·73 ± 0·063 (3)
85 days	M	1·01	0·52		0·40 ± 0·018 (4)	0·73 ± 0·028 (4)
	F	0·94	0·48		0·49 ± 0·022 (4)	0·86 ± 0·028 (4)
120–200 days	M	1·08	0·55	0·48 ± 0·014 (9)	0·40 ± 0·015 (9)	0·76 ± 0·026 (5)
	F	1·00	0·51	0·54 ± 0·019 (9)	0·48 ± 0·013 (9)	0·89 ± 0·030 (5)

* Of approximate length 25–30 mm. The number of determinations is given in brackets.

(Table 1). At the age of 45 days there was no difference between male and female rats. The increase of serotonin in the brain of female rats began between 65 and 85 days after birth when the sexual glands are fully active.

In preliminary experiments it had been observed that oestradiol (15 μg/g per day for 7 days) increased the brain serotonin content both in young female rats and adult male rats. On the contrary a treatment with testosterone (2 mg/g per day for 7 days) did not change the brain serotonin level in female or young male rats.

The differences observed suggest a role for oestrogens in regulating brain serotonin. This should be taken into account in evaluating the effects of psychotropic drugs on rat brain serotonin.

Institute of Pharmacology
University of Milano (Italy)

R. KATO*

REFERENCE

BOGDANSKI D. F., PLETSCHER A., BRODIE B. B. and UDENFRIEND S. (1957) *J. Pharmacol.* **117,** 82.

* Research Fellow from Keio University, Tokyo, Japan

VOL. 5 1959/6

γ-Aminobutyric acid and seizure susceptibility in areas of normal cat brain cortex*

(*Received* 3 *September* 1959)

In mammalian organisms, γ-aminobutyric acid (GABA) and glutamic acid decarboxylase (GAD) are found exclusively in the central nervous system (CNS), predominantly in the gray matter (Roberts *et al.*, 1951). Hydrazides depress the levels of GABA and GAD and at the same time increase seizure susceptibility (Killam, 1957; Killam and Bain, 1957). GABA is known to have inhibitory effects in various biologically excitable systems (Elliott and Jasper, 1959) and, when applied directly to the exposed cortex of cats, it has stopped thiosemicarbazide-induced seizure activity (Dasgupta *et al.*, 1958). Elevated levels of cerebral GABA produced by the administration of suitable doses of hydroxylamine appeared to have some sedative action in rats (Baxter and Roberts, 1959) and reduced susceptibility to electrically-induced seizures in cortical areas of cat's brain (Eidelberg *et al.*, 1959).

In view of these findings it appeared desirable to examine the relationship between normally occurring levels of GABA in different areas of the cerebral cortex and the seizure susceptibility of these areas.

METHODS

Six adult cats were used. The susceptibility of cortical areas to seizure was tested by a procedure similar to that described by Garner and French (1958). Electrical stimulation was applied locally through a pair of silver ball electrodes coated with silver chloride and resting on the cortical area being tested. Seizure thresholds were defined as the voltage required to elicit consistent and reproducible afterdischarges in the local electrocorticogram (ECG) after the application of a 5-sec train of rectangular bidirectional pulses, each lasting 0·1 msec. Threshold voltages and currents were checked on a calibrated oscilloscope at the end of each determination. A rest period of at least 5 min duration was maintained between the induction of each seizure.

Brain areas were sampled for biochemical study one hr after the last seizure threshold determination. At this time normal ECG patterns were found in all cortical areas. Identical areas of brain also were excised from cats which had received no electrical stimulation at any time. GABA in different cortical areas was measured with an adaptation (Baxter and Roberts, 1959) of the enzymic technique described by Jakoby and Scott (1959).

RESULTS

Measurements of seizure susceptibility in different areas of the cortex showed more than a threefold variation (Table 1). These observations are in agreement with those of Garner and French (1958). On the basis of fresh brain weight, all of the cortical areas contained about the same level of GABA. The hippocampus is known to have an exceptionally low seizure threshold requiring only the mechanical stimulation of exploring electrodes to fire convulsive afterdischarges (Green and Naquet, 1957). The level of GABA in the hippocampus was higher than in any of the other areas tested.

At the time of sampling, there was no difference between the levels of GABA in cortical areas of normal cats receiving no electrical stimulation, and those in which electrical stimulation had been used one hour previously for the determination of seizure thresholds. In normal, untreated cats the level of GABA (expressed as μg of GABA per g wet wt of tissue) ranged from 160 to 200 for sigmoid gyrus, 160 to 220 for posterior ectosylvian gyrus and 160 to 210 for the suprasylvian gyrus. These values are in good agreement with those presented in Table 1. Individual determinations from posterior marginal gyrus and hippocampus in untreated animals also fell within the range shown for these areas in Table 1. The method of testing for seizure threshold therefore did not appear to affect the level of GABA in these specific cortical areas at the time of sampling.

These results appear to rule out a direct relationship between seizure susceptibility and the naturally occurring level of GABA in any specific area of the cat brain cortex.

Roberts *et al.* (1958) have suggested that the balance between the 'GABA system' and the 'acetylcholine system', rather than the absolute amount of any single substance or enzyme activity might be important in the regulation of activity in a given region of the CNS. While the 'acetylcholine

* This work was supported in part by grant No. 1615 from the National Institute of Neurological Diseases and Blindness, U.S.P.H.S.

system' is only one of a number of systems which might be involved in such a balance with GABA, a concept of this nature could adequately account for both the lack of correlation between seizure susceptibility and the levels of GABA in the normal animal (as shown in Table 1) and the changes in seizure susceptibility which were observed when the levels of GABA were altered through the administration of hydrazides (KILLAM and BAIN, 1957) and hydroxylamine (EIDELBERG *et al.*, 1959).

TABLE 1.—GABA AND SEIZURE SUSCEPTIBILITY IN CAT BRAIN CORTEX AND HIPPOCAMPUS

Brain area	GABA μg/g of tissue*		Relative seizure susceptibility†
	Mean	Range	
Posterior marginal gyrus	168	120–230	+
Sigmoid gyrus	173	150–210	++
Posterior ectosylvian gyrus	167	120–210	++
Suprasylvian gyrus (3 areas)	174	110–210	+++
Hippocampus	234	160–300	++++

* This is the fresh wet weight of the brain tissue.

† The relative seizure susceptibility corresponded to the following voltage thresholds: + = 9–14 v, ++ = 5–8 v, and +++ = 1–4 v. Mechanical stimulation only was required to exceed the seizure threshold of the hippocampus.

The same stimulating electrodes and interelectrode distance were used for all cats. In any experiment the current per pulse was consistent with voltage values and thresholds were close to identical in symmetrical loci of both hemispheres. However, due to variability in impedance in the electrode–brain system the current output per pulse varied from one experiment to another.

Seizure susceptibility for any specific brain area under normal conditions might be unrelated to absolute levels of GABA but reflect only the point of balance with other systems, while pharmacologically induced changes in the level of GABA might alter such a balance and lead to changes in seizure susceptibility.

SUMMARY

Levels of free or easily extractable γ-aminobutyric acid and susceptibility to electrically induced seizures have been measured in areas of the cat brain cortex.

Although *changes* in levels of γ-aminobutyric acid in several species are accompanied by profound alterations in susceptibility to seizures, there is no direct relationship between *normal* levels of GABA and seizure susceptibility in various cerebral areas of the cat.

Department of Biochemistry
City of Hope Medical Center
Medical Research Institute
Duarte, California

CLAUDE F. BAXTER
EUGENE ROBERTS

Department of Neurophysiology
Long Beach Veterans Hospital
Long Beach, California

EDUARDO EIDELBERG

REFERENCES

BAXTER C. F. and ROBERTS E. (1959) *Proc. Soc. exp. Biol., N.Y.* **101,** 811.
DASGUPTA S. R., KILLAM E. K. and KILLAM K. F. (1958) *J. Pharmacol.* **122,** 16A.
EIDELBERG E., BAXTER C. F., ROBERTS E., SALDIAS C. A. and FRENCH J. D. (1959) *Proc. Soc. exp. Biol., N.Y.* **101,** 815.
ELLIOTT K. A. C. and JASPER H. J. (1959) *Physiol. Rev.* **39,** 383.
GARNER J. and FRENCH J. D. (1958) *Arch. Neurol. Psychiat., Chicago* **80,** 675.

VOL. 5 1959/6

GREEN J. D. and NAQUET R. (1957) *First International Congress of Neurological Sciences*, p. 225.
JAKOBY W. B. and SCOTT E. M. (1959) *J. biol. Chem.* **234**, 937.
KILLAM K. F. (1957) *J. Pharmacol.* **119**, 263.
KILLAM K. F. and BAIN J. A. (1957) *J. Pharmacol.* **119**, 255.
ROBERTS E., HARMAN P. J. and FRANKEL S. (1951) *Proc. Soc. exp. Biol., N.Y.* **78**, 799.
ROBERTS E., ROTHSTEIN M. and BAXTER C. F. (1958) *Proc. Soc. exp. Biol., N.Y.* **97**, 796.

OL.
S
9/60

Journal of Neurochemistry, 1960, Vol. 5, p. 206. Pergamon Press Ltd. Printed in Northern Ireland

Papers to be published in future issues

H. H. Hess and A. Pope: Intralaminar distribution of cytochrome oxidase activity in human frontal isocortex.

A. F. Harris and A. Saifer: The metachromasy of strandin.

N. M. Sulkin: The distribution of mucopolysaccharides in the cytoplasm of vertebrate cells.

C. F. Emanuel and I. L. Chaikoff: Desoxyribonucleic acids of central nervous system, kidney and spleen: a comparison of some chemical and physical properties.

W. G. van der Kloot: Factor S—a substance which excites crustacean muscle.

L. H. Cohen and W. K. Noell: Glucose catabolism of rabbit retina before and after development of visual function.

K. Yagi, T. Ozawa, M. Ando and T. Nagatsu: The effect of falvin adenine dinucleotide on the EEG, modified by chlorpromazine.

G. Porcellati and B. Curti: Proteinase activity of peripheral nerves during Wallerian degeneration.

M. Kurokawa: Metabolic consequences of localized application of electrical pulses to sections of cerebral white tissue.

C. W. M. Adams and A. N. Davison: The form in which cholesterol occurs in the adult CNS.

H. Sachs: Vasopressin biosynthesis—I. *In vivo* studies.

B. Hallgren and P. Sourander: The non-haemin iron in the cerebral cortex in Alzheimer's disease.

A. Geiger, N. Horvath and Y. Kawakita: The incorporation of ^{14}C derived from glucose into the proteins of the brain cortex, at rest and during activity.

A. Geiger, Y. Kawakita and S. S. Barkulis: Major pathways of glucose utilisation in the brain in brain perfusion experiments *in vivo* and *in situ*.

S. S. Barkulis, A. Geiger, Y. Kawakita and V. Aguilar: A study of the incorporation of ^{14}C derived from glucose into the free amino acids of the brain cortex.

T. Samorajski: DPN-diaphorase in nervous tissue.

R. Siminoff and P. R. Saunders: Effect of morphine on the uptake and oxidation of glucose by rabbit brain.

P. Hochstein and G. Cohen: The inhibitory effects of quinones and dihydric phenols on glucose metabolism in sub-cellular systems of brain.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 207 to 217. Pergamon Press Ltd. Printed in Northern Ireland

INTRALAMINAR DISTRIBUTION OF CYTOCHROME OXIDASE ACTIVITY IN HUMAN FRONTAL ISOCORTEX*

HELEN H. HESS† and ALFRED POPE
The McLean Hospital Research Laboratory, Waverley, Mass. and
the Department of Neuropathology, Harvard Medical School,
Boston, Mass.

(*Received* 26 *March* 1959)

OL.
5
9/60

IN THIS laboratory, the principles and techniques of quantitative histochemistry (LINDERSTRØM-LANG, 1939) are being used to study the biochemical architecture of human cerebral cortex. The immediate aim has been to determine relationships between the intracortical distributions of certain enzymes, representative of different metabolic pathways, and the histological structure of the cortex. The long range objectives are to establish a matrix of biochemical knowledge concerning normal human cortex and to investigate possible quantitative histochemical changes in certain nervous and mental diseases.

Previous reports have described the intralaminar distributions of acetylcholinesterase (POPE, CAVENESS and LIVINGSTON, 1952) and L-alanylglycine dipeptidase (POPE, 1959) activities in cortical biopsy specimens excised during frontal lobotomy. A similar investigation has been carried out upon the intracortical distribution of cytochrome oxidase, the results of which have already been presented in preliminary form (POPE and HESS, 1954; POPE, HESS and ALLEN, 1957). In this article, the details of this study are described in full.

Cytochrome oxidase was selected for study because of its critical importance in brain oxidative metabolism as mediator for electron transport between cytochrome *c* and oxygen.‡ Its activity is a simple and direct index of cortical oxidative capacity. Since it is readily inactivated, its assay in freshly excised tissue is desirable. For its assay, a microspectrophotometric method was developed (HESS and POPE, 1953), applicable to microgram samples of human cortex. This method has been used in conjunction with a frozen section sampling technique which provides samples whose dry weight, anatomical localization, and histological composition can readily be determined, so that the intracortical profile of the enzyme may be charted. These techniques have been used previously (POPE, HESS, WARE and THOMSON, 1956) to study the distribution of cytochrome oxidase in the layers of rat somatosensory isocortex. In this tissue, oxidase activity was high throughout the first five layers,

* This investigation was supported (in part) by Research Grant MH-295 from the National Institute of Mental Health and (in part) by Research Grant B-361 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, United States Public Health Service.

† Formerly Postdoctorate Research Fellow of the National Institute of Mental Health, National Institutes of Health, United States Public Health Service.

‡ The term cytochrome oxidase will be used to include cytochromes *a* and a_3.

The importance of oxidases other than cytochrome oxidase in brain metabolism is not known, but is probably small. NEUFELD *et al.* (1958) reported that the ratio of units of peroxidise to units of cytochrome oxidase in rat brain was 0·12, smaller than in most tissues.

but especially so in layer IIIb, as well as in layers IIa and either Vb or Vc; activity then declined rapidly through layer VI to values in white matter which were one fourth or less of the mean activity in grey matter. The distribution pattern of cytochrome oxidase in human frontal cortex has been found to be essentially similar, although the tendency for activity in layer III to exceed that in layers I and V is even more pronounced. As in rat cortex, the intracortical distribution is consistent with localization of cytochrome oxidase predominantly in neurons and their processes.

MATERIAL AND METHODS

The cortical biopsy specimens were obtained from patients undergoing prefrontal lobotomy for various forms of mental disease.* The specimen was removed from that portion of the frontal lobe designated Area 9 by Brodmann, Area FD_m by Von Economo and Koskinas (VON BONIN, 1950) and Area IEfs by BAILEY and VON BONIN (1951). A description of the technique used in excising the biopsy specimens and illustrations of the cyto- and myeloarchitecture of Area 9 have been given previously (POPE *et al.*, 1952). The specimens consisted of homotypical, eugranular frontal isocortex, corresponding with descriptions by the above authors as well as those by METTLER (1949) and by CONEL (1951).

The biopsy specimen was refrigerated immediately after its removal and during transport to the McLean Hospital Research Laboratory. Upon receipt, a cortical block was excised at once for enzyme study and frozen in a dry ice box. Remaining parts of the specimen were used for neuropathological study.

For determination of the cytoarchitectural distribution of cytochrome oxidase activity, the block of frozen cortex was sampled and sectioned in a cold room at $-12°$ according to a method in use in this laboratory (POPE *et al.*, 1952), which allows reasonably accurate identification of the histological composition and subpial depth of the samples being assayed. From the cortical block, a cylinder, 2 mm in diameter, was punched out, oriented with its long axis perpendicular to the free surface of the cortex. The frozen cylinder was mounted on a rotary microtome and cut serially from pia to white matter into slices 40 μ in thickness. These were studied in consecutive groups, each containing four sections: the first section for staining with buffered thionine (WINDLE *et al.*, 1943); the second for cytochrome oxidase assay by microspectrophotometry (HESS and POPE, 1953); the third for dry weight determination by means of a quartz fibre microbalance; while the fourth, in this instance, was discarded. Each slice for enzyme assay was placed in a chilled ground-glass microhomogenizer and kept at $-12°$ until it was to be homogenized. After transfer to a cold room at 4°, the slice was homogenized in 30 μl of 0·75% sodium deoxycholate for one min, with the homogenizer partly immersed in an ice–water bath. The homogenate was transferred to a micro test tube and centrifuged 10 min at 2800 rev/min at 4°. The clear supernatant fluid was used immediately for enzyme assay in an air conditioned room at 22°. The substrate solution consisted of $2{\cdot}4 \times 10^{-5}$ M-reduced cytochrome *c* in phosphate buffer, pH 7·1. Of the substrate solution, 65 μl were pipetted into a quartz microcuvette. Five μl of enzyme solution (tissue equivalent, 5–6 μg dry weight) were then added, and the solution was mixed. The rate of decrease in the optical density of the reduced cytochrome *c* at 550 mμ was observed at 15 sec intervals with a Beckman spectrophotometer, adapted for microtechniques as described by LOWRY and BESSEY (1946). The rate of change of optical density was determined during the initial phase of the reaction, which was often as long as three min. Since cytochrome oxidase is known to lose activity rapidly in aqueous solution at room temperature, the homogenate and supernatant fluid were refrigerated at 4° except when the latter was actually being assayed at 22°. This precaution, together with the brevity of the assay, guarded against deterioration of this sensitive enzyme during its study. All slices were assayed within 8 hr of being sectioned.

* All of the patients were lobotomized for various forms of mental illness at the Massachusetts Mental Health Center (Boston Psychopathic Hospital). The authors are deeply indebted to Dr. H. SOLOMON, Director, and Dr. M. GREENBLATT, Assistant Superintendent and Director of Research and Laboratories at the Massachusetts Mental Health Center, and to Dr. J. POPPEN and associates of the Lahey Clinic Neurosurgical Service for their invaluable co-operation and assistance in making available the cortical biopsy specimens.

In a subsequent article, an attempt will be made to correlate the results of these studies with the pathological and psychiatric information obtained upon this patient group.

VOL. 5 1959/6

The following additional precautions were observed because of the extreme sensitivity of cytochrome oxidase to poisoning by heavy metals and of cytochrome *c* to oxidation: glass-redistilled water was universally used; in cleaning glassware, dichromate–sulphuric acid mixture was avoided; instead, either 20% nitric acid or a mixture of equal volumes of concentrated nitric and sulphuric acids was used; micro test tubes and transfer pipettes (made from pyrex glass tubing, 3 mm inner diameter) were washed in Paragon or detergent solution, rinsed in tap and distilled water, boiled in redistilled water and discarded after use; microhomogenizers were washed in the same way, except that periodically they were treated with hot 20% nitric acid.

For each experiment, the cytochrome oxidase activities, expressed as change in optical density/min per μg dry weight, were plotted in relation to the theoretical subpial depth of the sample assayed and to the observed thicknesses of the cytoarchitectonic layers. The layer thicknesses were estimated first by examination of the histological control sections sampled at fixed intervals from the experimental cylinder. These results were correlated with direct measurements (the calibrated stage of a Leitz Ortholux microscope being used) upon vertical 20 μ thick frozen sections cut immediately adjacent to the site of the cylinder and stained with reduced silver.* Since the first section was discarded and the second used for histological reference, the initial enzyme sample centered about a depth of 100 μ and succeeding ones at 160 μ intervals thereafter. The average thickness of human frontal cortex in these biopsy specimens was 2745 μ. The sampling plan, therefore, yielded 15–19 samples within the grey matter; whenever possible, samples were also cut from the immediately subjacent white matter.

RESULTS

Presentation and analysis of the results of such experiments as these are made difficult by the complexity and variability of cortical histological structure and the wide individual variations in activities of enzymes in human population groups (WILLIAMS, 1956). Different aspects of the factors involved, the limitations of standard statistical treatment, and the reasons for using the simple form of presentation and analysis herewith employed have been discussed at length in previous communications (POPE *et al.*, 1956; HESS and POPE, 1959).

The cytochrome oxidase activities in ten biopsy specimens at standard subpial depths are presented in Table 1. The mean activities at these intracortical levels are shown in Fig. 1 in relation to the cytoarchitecture of human frontal cortex.

The composite pattern (Fig. 1) illustrates that in human frontal cortex cytochrome oxidase activity is greatest in the supragranular layers and progressively declines through the infragranular layers to low values in white matter. In seven of the ten cortices, the highest values for cytochrome oxidase were in layer III, in either sublayer IIIb or IIIc. In two of the remaining experiments, peak values in layers III and IV were about equal, whereas in the other the maximal activity was located at the junction of layers IIIc and IV. Activity in layer III was greater than that in layer I in eight of the ten experiments, in one instance it was the same, whereas in the remaining experiment it was slightly lower. In layer V, the activity was variable, although in six of the ten specimens values in the upper part of layer V were higher than in the remainder. In all instances in which sufficient data were obtained (9 of the 10 experiments), the activity in layer VIa was greater than in layer VIb, and in six of the ten experiments a small spike of activity occurred in layer VIa. In all experiments in which white matter was sampled, the activities were lower than those in layer VIb and less than 20 per cent of those in sublayers IIIb and IIIc. Thus, although the absolute values of oxidase activity at a given cortical level vary from one specimen to

* We are indebted to Mrs. MARIE ROSE for the technical preparation of the histological material used in this study.

TABLE I.—CYTOCHROME OXIDASE ACTIVITY IN RELATION TO CYTOARCHITECTURE OF HUMAN FRONTAL ISOCORTEX

Series No.	Depth, μ	Layer	Cytochrome oxidase activity (Δ_D/μg dry wt. per min $\times 10^3$)												
			Exp. 1 H-3	Exp. 2 H-9	Exp. 3 H-10	Exp. 4 H-13	Exp. 5 H-17	Exp. 6 H-19	Exp. 7 H-20	Exp. 8 H-21	Exp. 9 H-22	Exp. 10 H-26	Series Mean	Layer Mean*	Layer SEM†
1	100	I	3·28	2·01	1·73	3·72	6·67	2·37	5·05	2·42	3·10	4·17	3·45	3·45	0·48
2	260	II	5·24	2·84	3·09	4·21	6·66	3·19	3·95	3·78	5·07	5·12	4·32	4·24	0·41
3	420		5·36	2·21	2·06	3·67	7·28	3·88	6·13	5·58	3·63	4·87	4·47		
4	580	a	3·59	3·83	2·70	3·50	6·55	3·75	5·93	4·17	4·60	5·09	4·37	4·47	0·40
5	740	III b	4·51	4·05	3·02	4·51	8·52	6·49	6·10	5·20	4·04	4·65	5·11	5·05	0·52
6	900		4·63	4·28	2·61	5·03	8·53	5·61	6·47	6·17	5·30	5·88	5·45		
7	1060	c	5·47	3·10	1·84	3·45	5·73	5·96	6·91	3·92	4·51	5·26	4·62	4·95	0·48
8	1220		5·43	3·36	2·22	4·03	6·03	5·98	6·55	4·72	5·40	5·50	4·92		
9	1380	IV	6·57	1·54	2·81	2·49	7·98	5·43	3·69	2·46	4·48	4·13	4·16	4·29	0·42
10	1540		4·17	3·16	3·24	3·63	6·72	4·68	3·50	5·52	5·27	2·96	4·29		
11	1700		3·78	2·70	3·08	2·17	6·24	3·86	3·72	3·39	3·82	5·07	3·78		
12	1860	V	4·30	2·46	2·06	1·76	4·55	3·18	3·88	2·32	3·51	2·98	3·10	3·64	0·35
13	2020		3·69	2·46	2·43	3·44	4·20	1·99	5·06	2·81	4·04	3·05	3·32		
14	2180		4·45	1·86	2·34	2·10	5·53	2·60	3·33	2·01	1·61	3·11	2·89		
15	2340	a	3·47	1·79	2·04	1·82	3·39		3·51	1·76	1·57	2·44	2·42	2·86	0·30
16	2500	VI	2·72	1·89	1·43	1·40	4·85		2·04		1·47	3·52	2·42		
17	2660	b		1·29		0·80	3·36		2·45		0·92	2·53	1·89	1·81	0·37
18	2820		1·17	1·13		0·88	1·23		1·90		0·92	1·67	1·27		
19	2980	WM	1·46	0·63		0·0			1·59			0·96	0·93	0·89	0·23
20	3140					0·0			0·96			0·87	0·61		
21	3300								0·89			0·0	0·45		
22	3460											0·24	0·24		
23	3620											0·24	0·24		
Mean for cortex ‡O_2 utilized in ml per gm wet wt per hr at 37°			4·42	2·45	2·42	2·77	6·05	4·21	4·60	3·75	4·00	3·84			
			2·52	1·40	1·38	1·58	3·45	2·40	2·62	2·14	2·28	2·19			

Mean oxygen consumption for the series: 2·20 ml ± S.D. 0·58; S.E. = 0·19.

* For each cortical specimen, the separate determinations within each layer or sublayer were combined to give a mean laminar value. From the ten mean values thus obtained for each layer, the layer mean and its standard error for the entire experimental group were calculated.

† The mean oxidase activity in white matter is significantly lower than the mean activity in any of the layers of cortex. The mean activities in sublayers IIIb and IIIc are significantly higher than those in layers I and VI. Statistical significance was calculated by using Student's t test for small samples (FISHER, 1946). When $P < 0{\cdot}05$, the results were accepted as indicating statistical significance.

‡ The oxygen uptake corresponding to the cytochrome c oxidized may be calculated as described below. First, the results in the Table may be converted to μmoles of cytochrome c oxidized/mg dry wt per hr by use of the following equation (HORECKER and HEPPEL, 1949):

$$\text{Cy Fe}^{+++} = \frac{d_R - d_t}{1{\cdot}96 \times 10^4}$$

where the light path is one cm, the concentration of cytochrome c is in moles/l., d_R is the optical density after complete reduction with $Na_2S_2O_4$, d_t is the optical density after time t, and $1{\cdot}96 \times 10^4$ is the difference between the molecular extinction coefficients for reduced and oxidized cytochrome c ($2{\cdot}80 \times 10^4$ and

VOL. 5 1959/6

another, a familial pattern is evident in the intracortical distributions of oxidase activity within the group of specimens.

The mean cortical cytochrome oxidase activities (converted to oxygen uptake, Table 1) are in good agreement with the mean rates of oxygen consumption reported

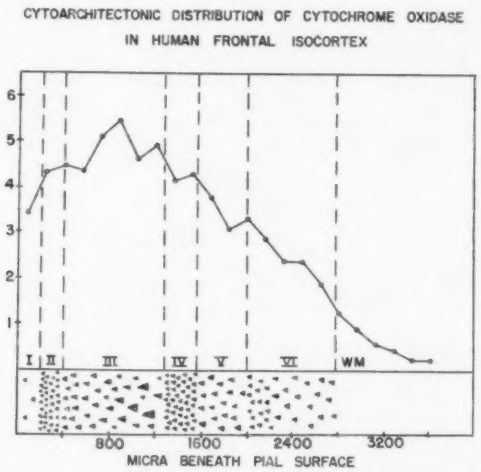


FIG. 1.—Intracortical distribution pattern of cytochrome oxidase in relation to subpial depths. Nissl cytoarchitecture and conventional laminar designations are depicted in sketch at base of Figure. In the present series of biopsies, average cortical thickness was 2745 μ. Layer I averaged 7·3 per cent; layer II, 7·7 per cent; layer III, 32·2 per cent; layer IV, 10·4 per cent; layer V, 15·9 per cent; and layer VI, 26·6 per cent of total width.
Vertical axis represents $\triangle$ O.D. per μg dry wt/min $\times 10^3$

by other investigators for human cortex (Table 2). From one biopsy specimen to another, however, considerable variability is apparent, not only in the oxidase activity at a particular level in the cortex but also in the mean cortical activity. This variability is to be ascribed to a number of factors which have been discussed at some length in a preceding article (HESS and POPE, 1959). One of the most important of these factors is the intraspecies variation in tissue levels of enzyme activity. Biochemical individuality, as evidenced by differences in blood and tissue levels of at

---

$0{\cdot}84 \times 10^4$ respectively). When the volume of the reacting solution is 70 μl (7×10^{-5} l.), it follows that:

μmoles Cyt. *c* oxidized/mg dry wt per hr at 22°

$$= \frac{(\Delta_D/\mu\text{g dry wt/min}) \times 10^3 \times 7 \times 10^{-5} \times 60 \times 10^6}{1{\cdot}96 \times 10^4}$$

$$= (\Delta_D/\mu\text{g dry wt/min}) \times 10^3 \times 0{\cdot}214$$

In calculating from this the equivalent volume of O_2 utilized/gm fresh wt per hour at 37° in grey and white matter, the following have been used:

Dry wt of grey matter = 17 per cent of fresh wt
Dry wt of white matter = 28 per cent of fresh wt
Q_{10} for the reaction = 2, i.e. $Q_{15} = 2{\cdot}8$

Hence, ml O_2 utilized/g fresh wt per hr at 37° =

$$(\Delta_D/\mu\text{g dry wt/min}) \times 10^3 \times 0{\cdot}214 \times \frac{22{\cdot}4}{4} \times 2{\cdot}8 \times (\%\text{ dry wt})$$

The figures in the Table are thus to be multiplied by the factor 0·570 for grey matter and 0·937 for white matter.

TABLE 2.—OXYGEN CONSUMPTION OF HUMAN CEREBRAL CORTEX AND WHITE MATTER
Manometric techniques were used to study the respiration of macro slices of brain, in most instances in a glucose–phosphate–saline medium. Departures from these conditions are noted in column 4.

Anatomical origin	μmoles O_2/g wet wt/hr at 37°	ml O_2/g wet wt/hr at 37°	Conditions	References
Cerebral cortex				
Frontal	57	1·28		McIlwain, Ayres and Forda (1952)
	84	1·88	Electrically stimulated	McIlwain, Ayres and Forda (1952)
Area 9		1·27		Elliott and Sutherland (1952)
		2·0	Krebs Ca^{++}-free medium with organic acids added.	Utena (1951)
Temporal	40	0·90		McIlwain, Ayres and Forda (1952)
	55	1·23	Electrically stimulated	McIlwain, Ayres and Forda (1952)
	53	1·19		Greengard and McIlwain (1955)
	98	2·20	Electrically stimulated	Greengard and McIlwain (1955)
	41	0·92		Brierley and McIlwain (1956)
	81	1·81	Electrically stimulated	Brierley and McIlwain (1956)
Mixed		1·2	Bicarbonate buffered	Elliott and Penfield (1948)
		2·0	Ca^{++} omitted	Elliott and Penfield (1948)
		1·2	Bicarbonate buffered	Heller and Elliott (1955)
		1·9	Ca^{++} omitted	Heller and Elliott (1955)
White matter				
Subcortical	25	0·56		Brierley and McIlwain (1956)
Temporal		0·75	Ca^{++} omitted	Heller and Elliott (1955)
Cerebellar		0·33	Ca^{++} omitted	Heller and Elliott (1955)

least some enzyme activities, appears to be especially characteristic of human subjects (Williams, 1956). Since in such groups knowledge and control of genetic, nutritional, and disease factors is rarely possible, variation of this kind is hardly surprising. The magnitude of variation in cytochrome oxidase activity in cortex as a whole is of about the same order as that observed in human brain oxygen consumption studied *in vitro* with fresh macro slices of cortex in the Warburg respirometer (Elliott and Sutherland, 1952), and *in vivo* by means of the nitrous oxide technique (Kety, 1957).

DISCUSSION

The results of these experiments will be discussed first in relation to the histological structure of human frontal isocortex. Secondly, the bearing of the results upon the vulnerability of human cortex to hypoxia will be briefly considered. Finally, the cytochrome oxidase activities obtained in the biopsy specimens will be compared with the results of other investigators who have studied the oxygen consumption of human brain *in vitro* and *in vivo*.

The most striking feature of the intracortical distribution profile of cytochrome oxidase in human frontal cortex is its resemblance to the corresponding curve obtained in rat somatosensory isocortex (Pope *et al.*, 1956). In rat cortex, cytochrome oxidase activity is well-sustained within layers I–V, although, relative to adjacent zones, it tends to be higher in layer IIa and in the middle or lower thirds of layers III and V. Through layer VI, the activity steadily declines, and in white matter values are obtained which are only one fifth of the highest values in grey matter. As discussed previously (Pope *et al.*, 1956), this distribution tends to parallel the intensity of the

VOL. 5 1959/6

histochemical indophenol oxidase reaction, and both can, in turn, be correlated with the total lengths of capillaries per unit volume. A comparison of this pattern with details of the neuroanatomical fine structure (LORENTE DE NÓ, 1949) shows that it is consistent with localization of cytochrome oxidase chiefly in neuronal cell bodies, their dendrites, and axonal terminals, structures which also are the principal intracortical loci of mitochondria.

The distribution pattern of cytochrome oxidase within the layers of human frontal isocortex differs little from that in rat somatosensory isocortex. The chief difference is that in human cortex the oxidase activity in layer III is more prominent in relation to the activities in layers I, V and VI than it is in rat cortex.

It seems probable that in human as in rat cortex, cytochrome oxidase activity is chiefly localized in neurons and their processes rather than in neuroglial cells. This is suggested first of all by the fact that the cytochrome oxidase activity of human cortical grey matter is three- or fivefold greater than that of subjacent white matter when expressed in terms of fresh or dry weight, respectively. Differential cell counts in Area 9 (POPE, 1959) show that, apart from an increase in oligodendroglia in layers V and VI and of astrocytes in layers I and IV, the number of neuroglia per unit volume varies relatively little from layer to layer. Moreover, in white matter, astrocytes are equally, if not more, abundant, whereas oligodendroglia are increased four- or fivefold over their numbers in grey matter. Since the total number of cells per unit volume is somewhat greater in subjacent white than in cortical grey matter, the oxidase activity of these glial cells must be appreciably less than that of cortical neurons. This conclusion is also consistent with the relatively low cytochrome oxidase activity shown by well-differentiated human gliomas (ALLEN, 1957).

Because differential centrifugation studies upon many tissues, including brain, have shown a very close and perhaps exclusive association of cytochrome oxidase with mitochondria (SCHNEIDER and HOGEBOOM, 1951; BRODY *et al.*, 1952), it seems reasonable to assume that the distribution of this enzyme in a tissue reflects the quantitative distribution of mitochondria within it. Accordingly, it may be concluded that the greatest amount of mitochondrial material is located in layers II, III, and IV of human frontal cortex, especially in sublayers IIIb and IIIc. Electron micrographs show that higher densities of mitochondria are found in most neurons and their larger processes than in oligodendroglia or astrocytes, or in axons (WINDLE, 1958). Their density within the perikaryon is unequal in neurons of different types. In addition to being distributed throughout most of the neuronal perikaryon, mitochondria are prominent in presynaptic terminals and at postsynaptic membranes, especially in dendrites (FERNÁNDES-MORÁN, 1957). It appears reasonable, therefore, to anticipate a greater population of mitochondria where large numbers of axons terminate upon medium to large neurons and their extensive dendrites. In Area 9, the largest cells are the prominent pyramids of sublayers IIIb and IIIc. It is probable that large numbers of axons terminate in relation to these cells. The high cytochrome oxidase activity of these sublayers may, therefore, reflect the presence of mitochondria accompanying abundant axodendritic connexions with neurons having themselves mitochondria-rich perikarya.

The lesser oxidative activity per unit dry weight found in layers I, V, and VI in human cortex can be related to certain of their histological features. Horizontal myelinated axons in layer I, and horizontal and especially vertical myelinated axons

in layers V and VI contribute significantly to the volume and dry weight in these layers. Also, layer I contains few neurons, all of which are small, and greater numbers of astrocytes than does layer III. Finally, the pyramids of layer V are smaller than those in layer III and probably have fewer axodendritic connexions.

The distribution of cytochrome oxidase activity within the layers of human frontal cortex thus suggests that the principal cytological sites of cortical oxidation are the neuronal cell bodies, dendrites, and axon terminals. This is in conformity with the results of ELLIOTT and HELLER (1957), who calculated that in human cortex 66–71 per cent of the respiration was contributed by neurons. Preliminary calculations of a similar nature, based upon the cytochrome oxidase activities in the present series of human cortical biopsy specimens and in human gliomas (ALLEN, 1957) suggest that as much as 90 per cent of the respiration of Area 9 cortex may be neuronal. ELLIOTT and HELLER (1957) estimated that in cat and dog cortex, neurons were responsible for 85 per cent of oxygen uptake. Similar conclusions were reported by KOREY and ORCHEN (1959) with respect to lamb cortex.

The intracortical distribution of cytochrome oxidase may be significant with respect to the peculiar vulnerability of the cortex to hypoxia. MEYER (1958) has reviewed the neuropathological changes in human brain following anoxic states produced by a variety of conditions and agents, and has discussed the problem of selective vulnerability. In general, a single, severe hypoxic episode leads to predominantly cortical lesions in man. Layer III is most often affected, and necrosis may be restricted to it in a laminar fashion. With more prolonged hypoxia, necrosis may occur in all cortical layers as well as in other brain areas. Layer III of human frontal cortex has a higher cytochrome oxidase activity than other layers, and hence, presumably, a higher rate of oxygen consumption. In this layer, the number of neurons per unit volume is less than it is in most of the other layers, but the cells are large and have extensive dendrites. The evidence indicates, therefore, that the oxygen requirement per minute per neuron is higher in layer III than at other intracortical levels. The somewhat greater vulnerability of this layer to brief, severe hypoxia is in accord with the idea that, under these conditions, those neurons having the highest rate of oxygen consumption/cell per minute will be the first to suffer irreversible structural changes. Further quantitative histochemical studies may be expected to define more clearly the role which the biochemical characteristics of certain parts of the brain play in their vulnerability to hypoxia.

The average cytochrome oxidase activity measured in human cortex is approximately ten per cent of that found in rat cortex. A lower activity was anticipated but such a large difference was somewhat surprising. The basal metabolic rate (in terms of kcal/kg body weight per hr) is only about one fifth as great in man as it is in the rat. However, although Q_{O_2}* values for many individual tissues, including brain, are in fact lower in larger than in smaller animal species (KREBS, 1950), tissue Q_{O_2} changes much less with average adult body weight than does the basal metabolic rate. ELLIOTT (1948) found the Q_{O_2} of slices of human cortex respiring in glucose–saline media to be one half that of rat cortex. The highest rates for rat cortex have been obtained with Krebs' Ca^{++}-free supplemented medium (KREBS, 1950), in which the average Q_{O_2} is 26 (4·4 ml O_2/g fresh wt. per hr at 37°). However, when the cytochrome oxidase activity of rat brain homogenate is measured manometrically

* Q_{O_2} will refer to microlitres O_2 consumed/mg dry wt. per hr, at 37°.

VOL. 5 1959/6

in a medium containing *p*-phenylenediamine and supplementary cytochrome *c*, the Q_{O_2} is 134 (ELLIOTT and GREIG, 1938). Similarly, the cytochrome oxidase activity of rat cortex, assayed by microspectrophotometry (HESS and POPE, 1953), is equivalent to a Q_{O_2} of 143. Apparently, unless exogenous cytochrome *c* is provided in the incubation medium, the cytochrome oxidase of rat brain functions at only a fraction of its maximal rate. In this tissue, oxidase activity seems to be present in excess (ELLIOTT and GREIG, 1938) and the concentration of cytochrome *c* may be the rate-limiting factor in cellular oxidations.

In human cortex, on the other hand, the cytochrome oxidase activity (expressed as oxygen uptake/g fresh wt., Table 1) is consistent with rates of cortical respiration reported by other investigators. The results of pertinent, comparable investigations upon the oxygen uptake of human brain in vitro have been summarized in Table 2. The mean cytochrome oxidase activity found in our series of biopsy specimens is equivalent to the maximal rate of respiration recorded in Table 2 when a Ca^{++}-free medium or electrical stimulation is employed. This suggests that an optimal concentration of cytochrome *c* in relation to cytochrome oxidase exists in human cortex. Since little or no excess of cytochrome oxidase seems to be present, this enzyme may be rate-limiting in cellular oxidations in this tissue.

Numerous *in vivo* determinations by means of the nitrous oxide method (KETY, 1957; KENNEDY, 1956) indicate that the oxygen consumption of the brain of normal adults in the resting state is $2{\cdot}1 \pm 0{\cdot}4$ ml/g fresh wt. per hr (3·5 ml/100 g per min). The results obtained by studies *in vitro* are in the right range to account for the resting respiration *in vivo*, especially if allowance is made for the possibility that some regions of grey matter may have a higher oxidative rate per g fresh wt. than does cerebral cortex. In this regard, it is noteworthy that in beef and dog brain cerebellar cortex and caudate nucleus exceed cerebral cortex in their rates of oxygen consumption per g fresh wt. (ELLIOTT, 1955). It is not known, however, to what extent the rate of oxygen uptake of the human brain increases during maximal activity such as occurs in convulsive seizures. If, as in the case of monkey brain (KETY, 1957), it is approximately doubled during a seizure, the demonstrable cytochrome oxidase activity would be insufficient to account for the increase.

All the methods of measurement of oxygen utilization *in vitro*, therefore, may have underestimated the potential oxidative capacity of human brain tissue. Several factors may have contributed to this. Cytochrome oxidase is an exceedingly sensitive enzyme, and the possibility that pharmacological agents or early autolytic changes may have affected its activity cannot be completely dismissed.* In this investigation and in those of others, the specimens studied were removed from patients given preoperative medication (morphine and atropine or related drugs) as well as an anaesthetic agent. In the present series, local anaesthetics were employed in two cases and sodium pentothal in the remainder. None of these agents has been shown to inhibit directly the activity of cytochrome oxidase, although some of them do inhibit the increased oxygen uptake induced by electrical stimulation (MCILWAIN, 1950, 1955). However, under the conditions of the enzyme assay, any drug present would have been greatly diluted, and its effect thereby reduced. The effect of storing the excised tissue at 0° for 30–45 min (during transport to the laboratory) is not known.

* The studies described in this paper were completed before the tranquilizing drugs such as chlorpromazine and reserpine came into use in psychiatry.

In homogenates standing at room temperature, oxidase activity is lost over a period of several hours at a rate which varies with pH; although the rate is slower at 0°, some inactivation occurs. In intact tissue, the rate of inactivation is slower than in homogenates.

In addition, the point may be raised that none of the samples of cortex used for this and other studies was obtained from normal adults. Studies *in vivo*, however, have not shown any consistent difference in the resting oxygen consumption of the brain in psychiatric or epileptic patients as compared with normal persons (KETY, 1957). This does not demonstrate, of course, that the same reserve capacity for increased respiration during heightened nervous activity exists in the two groups, nor does it rule out the possibility that in such patients an abnormality in respiration may occur only in a particular anatomical region of the brain.

SUMMARY

The methods of quantitative histochemistry have been used to determine the distribution of cytochrome oxidase activity within the architectonic layers of human cerebral cortex in biopsy specimens removed during frontal lobotomy.

Cytochrome oxidase activity/unit dry wt. of cortex was greatest in layers II, III, and IV, especially in sublayers IIIb and IIIc. In layers I and VI, the activity was significantly lower, whereas in subcortical white matter, the activity was only one fifth as great as that in layer III. The intralaminar pattern of cytochrome oxidase presumably reflects the distribution of mitochondria and is consistent with localization of the enzyme chiefly in the cell bodies and dendrites of the neurons and in axon terminals.

The mean cortical cytochrome oxidase activities in these specimens were consistent with those to be expected from previous investigations upon the *in vitro* oxygen consumption of human cortex.

The possible significance of these results for the problem of cortical vulnerability to hypoxia has been discussed.

REFERENCES

ALLEN J. N. (1957) *J. Neurochem.* **2**, 37.

BAILEY P. and BONIN VON G. (1951) *The Isocortex of Man.* University of Illinois Press, Urbana.

BONIN VON G. (1950) *Essay on the Cerebral Cortex.* Thomas, Springfield, Ill.

BRIERLEY J. B. and MCILWAIN H. (1956) *J. Neurochem.* **1**, 109.

BRODY T. M., WANG R. I. H. and BAIN J. A. (1952) *J. biol. Chem.* **198**, 821.

CONEL J. L. (1951) *Postnatal Development of Human Cerebral Cortex*, Vol. IV, *Cortex of Six Month Infant.* Harvard University Press, Cambridge.

ELLIOTT H. W. and SUTHERLAND V. C. (1952) *J. cell. comp. Anat.* **40**, 221.

ELLIOTT K. A. C. (1948) *J. Neurophysiol.* **11**, 473.

ELLIOTT K. A. C. (1955) *in Neurochemistry* (Edited by ELLIOTT K. A. C., PAGE I. and QUASTEL J. H.) p. 53. Thomas, Springfield.

ELLIOTT K. A. C. and GREIG M. E. (1938) *Biochem. J.* **32**, 1407.

ELLIOTT K. A. C. and HELLER I. H. (1957) *in Metabolism of the Nervous System* (Edited by RICHTER D). p. 286. Pergamon Press, London.

ELLIOTT K. A. C. and PENFIELD W. (1948) *J. Neurophysiol.* **11**, 485.

FERNÁNDES-MORÁN H. (1957) *in Metabolism of the Nervous System* (Edited by RICHTER D.) p. 1. Pergamon Press, London.

FISHER R. A. (1946) *Statistical Methods for Research Workers.* Oliver and Boyd, London.

GREENGARD P. and MCILWAIN H. (1955) *in Biochemistry of the Developing Nervous System* (Edited by WAELSCH H.) p. 251. Academic Press, New York.

VOL.
5
1959/6

HELLER I. H. and ELLIOTT K. A. C. (1955) *Canad. J. Biochem. Physiol.* **33,** 395.
HESS H. H. and POPE A. (1953) *J. biol. Chem.* **204,** 295.
HESS H. H. and POPE A. (1959) *J. Neurochem.* **3,** 287.
HORECKER B. L. and HEPPEL L. A. (1949) *J. biol. Chem.* **178,** 683.
KENNEDY C. (1956) In *Progress in Neurobiology I. Neurochemistry* (Edited by KOREY S. R. and NURNBERGER J. I.) p. 239. Hoeber-Harper, New York.
KETY S. S. (1957) In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 221. Pergamon Press, London.
KOREY S. R. and ORCHEN M. (1959) *J. Neurochem.* **3,** 277.
KREBS H. A. (1950) *Biochim. biophys. acta* **4,** 249.
LINDERSTRØM-LANG K. (1939) *Harvey Lect.* **34,** 214.
LORENTE DE NÓ R. (1949) In *Physiology of the Nervous System* (Edited by FULTON J. F.) (3rd. Ed.) Chapter XV. Oxford University Press, New York.
LOWRY O. H. and BESSEY O. A. (1946) *J. biol. Chem.* **163,** 633.
MCILWAIN H. (1950) *Brit. med. Bull.* **6,** 301.
MCILWAIN H. (1955) *Biochemistry and the Central Nervous System.* Little, Brown, Boston.
MCILWAIN H., AYRES P. S. W. and FORDA O. (1952) *J. mental Sci.* **98,** 265.
METTLER F. A. (1949) In *Selective Partial Ablations of the Frontal Cortex* (Edited by METTLER F. A.) p. 48. Hoeber, New York.
MEYER A. (1958) In *Neuropathology* (Edited by GREENFIELD J. G., BLACKWOOD W., MCMENEMEY W. H., MEYER A. and NORMAN R. M.) p. 230. Arnold, London.
NEUFELD H. A., LEVAY A. N., LUCAS F. V., MARTIN A. P. and STOTZ E. (1958) *J. biol. Chem.* **233,** 209.
POPE A. (1959) *J. Neurochem.* **4,** 31.
POPE A., CAVENESS W. and LIVINGSTON K. E. (1952) *Arch. Neurol. Psychiat., Chicago* **68,** 425.
POPE A. and HESS H. H. (1954) *Fed. Proc.* **13,** 275.
POPE A., HESS H. H. and ALLEN J. N. (1957) In *Progress in Neurobiology II. Ultrastructure and Cellular Chemistry of Neural Tissue* (Edited by WAELSCH H.) p. 182. Hoeber-Harper, New York.
POPE A., HESS H. H., WARE J. R. and THOMSON R. H. (1956) *J. Neurophysiol.* **19,** 259.
SCHNEIDER W. C. and HOGEBOOM G. H. (1951) *Cancer Res* **11,** 1.
UTENA H. (1951) *Psychiat. Neurol. Japon.* **52,** 204.
WILLIAMS R. J. (1956) *Biochemical Individuality.* Wiley, New York.
WINDLE W. F. (Editor) (1958) *The Biology of Neuroglia.* Thomas, Springfield, Ill.
WINDLE W. F., RHINES R. and RANKIN S. (1943) *Stain Technol.* **18,** 77.

Journal of Neurochemistry, 1960, Vol. 5, pp. 218–230. Pergamon Press Ltd. Printed in Northern Ireland

THE METACHROMASY OF STRANDIN*

ALFRED F. HARRIS and ABRAHAM SAIFER
Biochemistry Department, Isaac Albert Research Institute
of the Jewish Chronic Disease Hospital Brooklyn 3, N.Y.

(*Received* 13 *April* 1959)

STRANDIN, a material of high molecular weight (mol. wt. $>$ 250,000) obtained from normal and diseased brain tissue (FOLCH, ARSOVE and MEATH, 1951; FOLCH, LEES and SLOANE-STANLEY, 1957), is found in highest concentration in gray matter and in large amounts in amaurotic familial idiocy. Although related to the cerebrosides, containing as it does fatty acid, sphingosine and hexose (glucose and galactose), it is more closely related to the gangliosides of Klenk, since it also contains hexosamine (galactosamine), neuraminic acid and probably amino acids. An interesting property of this complex mucolipid is its solubility in water (FOLCH and LEES, 1959). The metachromasy of strandin has been noted by ROSENBERG and CHARGAFF (1959). BRANTE (1957) has described the metachromasy of the ganglioside derived from a case of gargoylism.

Metachromasy may be defined histologically as the capacity of certain dyes to stain tissues with a different hue from that of the original dye. As measured photometrically with a chemically pure chromotrope and a solution of the dye (LISON, 1935), the reaction manifests itself predominantly as a shift in the absorption spectrum towards shorter wave-lengths. The phenomenon is manifested by a number of cationic dyes, namely, azine, azo, oxazine, phenylmethane, quinoline and thiazine (KELLY, 1956*a*). It is to the last group, in particular toluidine blue O and azine A, that the greatest attention has been directed.

The work of a number of investigators has established some of the conditions which determine metachromatic behavior. Among these are the ratio of dye to chromotrope, temperature, pH, and the effects of proteins, salts and organic solvents (HALE, 1957). It is our present purpose to present a study of the metachromasy of strandin, the effect of the various factors mentioned above on the character of the reaction being taken into consideration.

MATERIALS AND METHODS

Materials

Strandin was prepared from cortical gray matter of beef brain by the extraction procedure of FOLCH, LEES and SLOANE-STANLEY (1957), and a stock solution containing 60 mg per 100 ml was prepared. This figure was arrived at by analysing the strandin preparation with the method for neuraminic acid of SAIFER and GERSTENFELD (1957) and assuming an average value of 30·5 mg of neuraminic acid per 100 mg of strandin (FOLCH and LEES, 1959). The analytical value for strandin concentration was corroborated by the Browning reaction (FOLCH and LEES, 1959).

Toluidine blue O was obtained from the National Aniline Division (NAD), Allied Chemical and Dye Corp., N.Y. It has a total dye content (TDC) of 94%. A stock solution was prepared containing 10 mg of dye (9·4 mg of pure dye) per 100 ml. The molarity of this stock solution is $2{\cdot}12 \times 10^{-4}$ M.

Other dyes used in this study were: Nile blue sulphate (NC, AD, TDC, 86%); acriflavine, N.F.

* Supported by a grant from the National Tay-Sachs' Association.

VOL.
5
1959/6

(Abbot Laboratories, North Chicago, Ill.); Janus green B (NAD, TDC, 71%); safranin O (Hartmann and Leddon Co., TDC, 86%), neutral red (NAD, TDC, 82%); crystal violet (NAD, TDC, 93%); and pinacyanol, which was obtained from K and K Laboratories, Long Island City, N.Y. These dyes were prepared as stock solutions containing 10 mg of dye per 100 ml of distilled water. Solutions of 0·2% were used to study the effect of various proteins on the metachromatic reaction of strandin. Protamine sulphate (salmine) C.P. was obtained from the Mann Research Laboratories, N.Y. Both pepsin and chymotrypsin were obtained from the Worthington Biochemical Corp., Freehold, N. J. Egg albumin was obtained from the Delta Chemical Works, New York. Bovine albumin was obtained from Pentex Incorporated, Kankakee, Ill.

All the metal salts and organic solvents employed in this study were of reagent grade.

Methods

The term 'working solution' refers to 1 ml of 0·0094% toluidine blue O (TBO) plus 1 ml of 0·06% strandin, diluted to a volume of 20 ml with distilled water. The TBO content of the working solution is $1{\cdot}06 \times 10^{-5}$ M, while the strandin concentration is 0·003%.

In measuring metachromasy with TBO, a modified Wiame ratio (WIAME, 1947), which we have designated as optical density ratio (ODR), was employed, where ODR is equal to (OD 550/OD 635). Measurements of the *loss* of metachromasy involved a determination of the change in ODR, i.e. Δ(ODR), which is given by the following equation,

$$\Delta(\mathrm{ODR}) = (\mathrm{ODR}_0 - \mathrm{ODR}_x)$$

where (ODR_0) represents the initial value of the ratio and (ODR_x) its value at any concentration x of the substance repressing metachromasy. Since the value of (ODR_x) will decrease with loss of metachromasy, Δ(ODR) will increase. Δ(ODR) was used in all such cases, except that involving the comparison between strandin and chondroitin sulphate (CSA), in regard to the effect of hydrogen ion concentration on their behavior.

pH measurements were made of each solution with a Beckman Model G pH meter and the optical density values were obtained with a Bausch and Lomb Spectronic 20 spectrophotometer.

RESULTS

Dye-chromotrope ratio

To study the effect of the dye-chromotrope ratio on the metachromasy of strandin, the concentration of strandin was varied at a constant dye concentration at room temperature (25·5°), with the results indicated in Fig. 1. With increasing concentration of chromotrope there is a gradual depression of the primary band (635 mμ) and the appearance of a β-band at 575 mμ at 12·0 mg/l. of strandin. This band becomes more pronounced at 30·0 mg/l. of strandin, where the maximum occurs at 560 mμ. At a strandin concentration of 60·0 mg/l., there is a loss of metachromasy, the β-band shifting to a wavelength of 580 mμ. This effect of loss of metachromasy produced by increasing the concentration of strandin, is best shown by the effect on the ODR (OD 550/OD 635) (Fig. 2); it is at intermediate concentrations that the most satisfactory evidence of metachromasy is found. The maximal value of ODR (at room temperature), 1·6, appears to fall between 30 mg/l. and 37 mg/l.

Temperature

The effect of various concentrations of strandin on the TBO absorption spectrum was determined at 6° (Fig. 3). The concentrations are the same as those indicated in Fig. 1, except that the 30·0 mg/l. concentration has been omitted. Three points are to be noted in connection with this curve:

(a) The spectrum of the pure dye has split into a diphasic curve which indicates the

possible appearance of two dye species. In addition the overall absorption has been depressed.

(b) At the 3·0 mg/l. concentration the curve has a diphasic form and also seems to maintain a fairly broad plateau at an OD maximum of 0·440 between 590 mμ and 620 mμ.

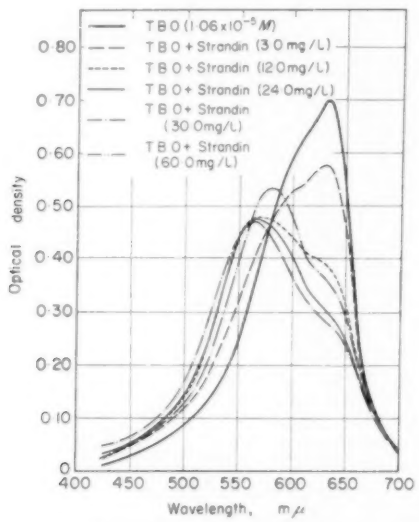


FIG. 1.—The effect of strandin on the absorption spectrum of toluidine blue O at 25·5°. Concentrations as indicated above.

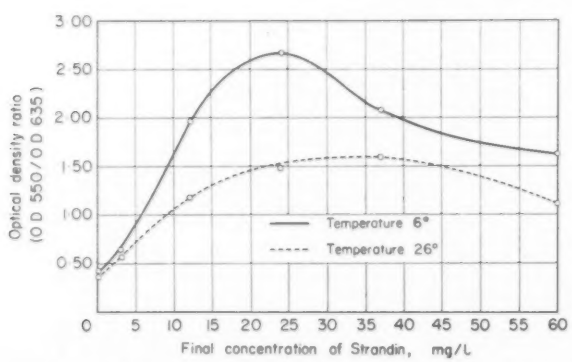


FIG. 2.—The effect of strandin concentration on the value of the optical density ratio at 6° and 26°. The concentration of toluidine blue O is $1{\cdot}06 \times 10^{-5}$ M.

(c) At a strandin concentration of 24·0 mg/l. the colour of the solution is a pronounced red-purple and the maximal OD exists at 550 mμ. We have indicated this as the wavelength characteristic of the metachromasy of this compound (i.e. the γ-band), 635 mμ being used as that characteristic of the normal form of the dye. Hence the ODR is based on the calculation (OD 550/OD 635). It is at this concentration that the diphasic nature of the curve is for all practical purposes completely lost. At higher concentrations of strandin (36 mg/l. to 60 mg/l.) there is a loss in metachromasy as is

VOL. 5 1959/6

the case at room temperature. This is indicated by the plot of the ODR as a function of strandin concentration (Fig. 2).

The effect of temperature is further demonstrated by the variation in ODR resulting from temperature change over the interval 17° to 45° (Fig. 4). The rate of loss of metachromasy is linear with respect to temperature over the interval studied.

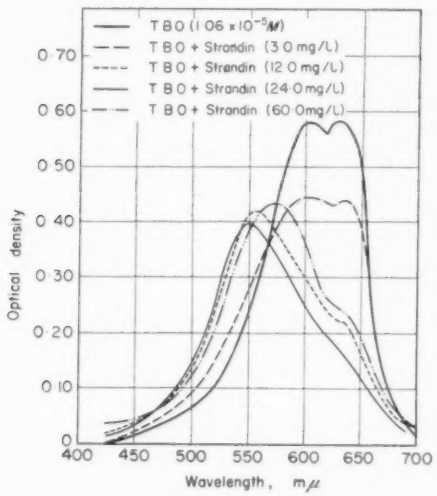


FIG. 3.—The effect of strandin on the absorption spectrum of toluidine blue O at 6°. Concentrations as indicated above.

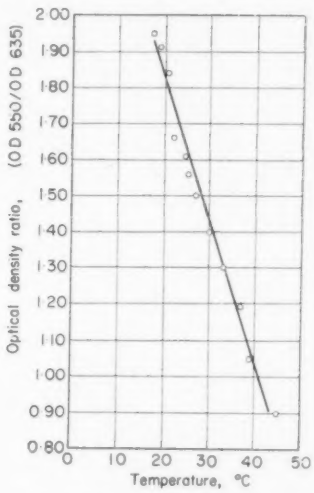


FIG. 4.—The effect of temperature on the metachromasy of the strandin-toluidine blue O complex. The concentration of toluidine blue O is $1{\cdot}06 \times 10^{-5}$ M and that of strandin is 24 mg/l.

Effect of pH *on metachromasy*

The working solution, prepared as described previously, was titrated with 0·1 N-HCl for the determination of ODR in the acid region, and with 0·1 N and 1·0 N-NaOH for its measurement in the alkaline range (Fig. 5). The metachromasy of the system rises in sigmoid fashion as the pH increases from 2·0 to 7·0. There is little or no change in the interval between 6·0 and 8·5. The curve undergoes a slight decline after pH 8·5 and continues in this direction until pH 10·3 is reached.

To shed further light on the nature of the reaction in the alkaline range, we titrated a solution of the dye alone ($1{\cdot}06 \times 10^{-5}$ M) with NaOH (0·01, 0·1 and 1·0 N) and plotted the values for the ODR as a function of pH (Fig. 5B). This curve shows a point of inflection at pH 10·3, the value of the ODR reaching 0·95 at pH 10·8; at pH 12·2 it would theoretically approach an intersection with the strandin–TBO curve, reaching, as it does, an ODR value of 1·5. In view of the complicating effect of alkali on the dye solution, our studies concerning the effect of pH on the metachromasy of strandin were discontinued at pH 10·3.

The metachromasy of the strandin–TBO system remains fairly constant in the pH interval between 6·0 and 8·5. This fact is of interest in considering the pH values of the solutions employed in the study of the quenching of metachromasy. Since these solutions have pH values in the range, 6·0–7·3, the effect of hydrogen ion may be either ignored, or, as in the case of $AlCl_3$ (pH 4·4), may be corrected for. The only

possibly deviant value, that of pepsin (pH 5·9), may be considered to lie within the range of pH stability.

There is a steady drop in the value of Δ(OD 635) with increase in pH (Fig. 6). The measurement and use of (ΔOD 635 mμ) as an index of metachromatic loss has been

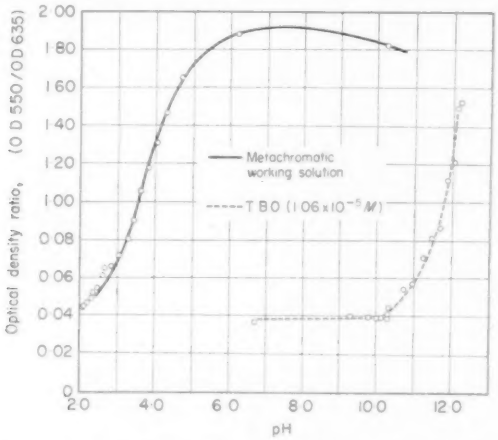


FIG. 5.—The effect of pH on the metachromasy of the strandin-toluidine blue O working solution (—) and on the optical density ratio of toluidine blue O ($1{\cdot}06 \times 10^{-5}$ M) (···). Solutions were titrated with HCl and NaOH as indicated in text.

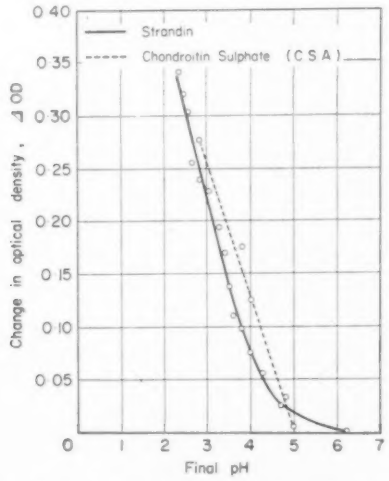


FIG. 6.—The effect of pH (acid range) on the metachromasy of chondroitin sulphate and of strandin. The working solution of chondroitin sulphate consists of a 20 ml solution containing 0·002% (w/v) of chondroitin sulphate and $7{\cdot}8 \times 10^{-6}$ M-toluidine blue O. The working solution of strandin is described in the text. Of the working solution, 20 ml were titrated with 0·1 N-HCl. Δ(OD) is the change in optical density at 635 mμ.

discussed in a previous publication (HARRIS and SOBEL, 1958). The change between pH 5 and 6 is extremely small and beyond 6·2 (6·2 to 8·0) little or no effect is to be observed. The effect is similar to that found for chondroitin sulphate (CSA) (HARRIS and SOBEL, 1958), except that the curve for strandin becomes non-linear at about pH 4·5.

The effect of protein

We studied the behavior of protamine sulphate, pepsin, chymotrypsin, egg albumin, and bovine albumin on the metachromasy of the strandin–TBO working solution. The results are indicated in Fig. 7 as loss of metachromasy (Δ (ODR)) as a function of final protein concentration. Protamine shows a change of 1·27 whereas the change for the other proteins ranges from 0·15 to 0·25. The temperature in these experiments averaged 25·5° and the final pH values ranged from 5·9 (pepsin) to 7·2 (egg albumin).

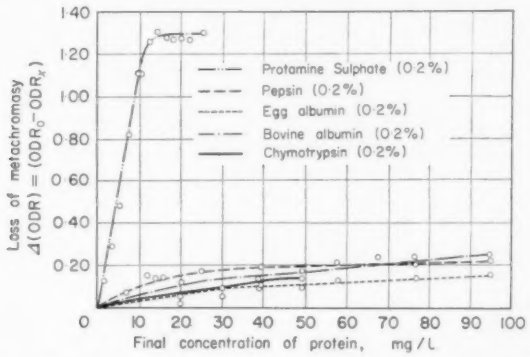


FIG. 7.—The effect of some proteins on the metachromasy of the strandin-toluidine blue O working solution (25·5°).

Thus the various proteins all cause a loss of metachromasy of the strandin–TBO mixture, the effect of protamine being by far the greatest and great enough to cause the spectrum of the solution to revert to a form practically equivalent to that of the original dye.

Effect of inorganic cations

The effect of various cations in repressing the metachromasy of strandin was measured by using Δ(ODR) as a function of final salt concentration. The results (Fig. 8) were all in the pH range from 6·0 to 6·8 with the exception of $AlCl_3$ (pH 4·4) for which suitable corrections were made. The figure indicates that the capacity to quench metachromasy is dependent on valency at lower salt concentrations. This effect, as in the case of CSA (HARRIS and SOBEL, 1958), follows the order:—

tervalent > bivalent > univalent

The tervalent salts attained their maximal level more rapidly, but reached about the same plateau value as the bivalent salts (Fig. 8). Salts not only affected the metachromasy of strandin, but also the spectrum of the dye in the absence of strandin (Table 1). Since high concentrations of some salts are known to cause metachromatic phenomena (MICHAELIS, 1947), it is not unreasonable to find that the absorption spectrum is affected to some extent at the relatively low concentrations employed in these experiments.

The effect of organic solvents

Since it is known that alcohol can destroy the metachromasy of histological preparations, we were interested in determining the effect of a number of alcohols on the

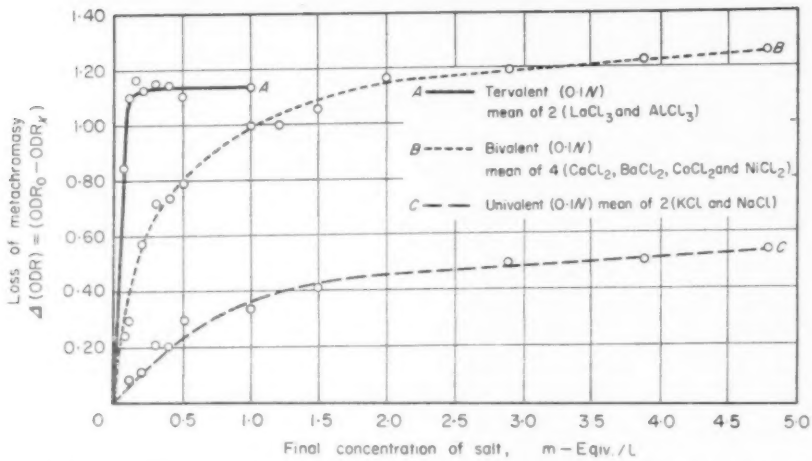


FIG. 8.—Comparison of the effects of salts (cations) in suppressing the metachromasy of the strandin-toluidine blue O working solution (25·5°).

TABLE 1.—INTERACTION OF SALTS WITH TBO ($1{\cdot}06 \times 10^{-5}$ M)

Salt	Amount	OD(635 mμ)	Δ(OD)*
Water	1 ml	0·660	—
0·1 N-KCl	1 ml	0·640	−0·020
0·1 N-NaCl	1 ml	0·640	−0·020
0·1 N-$CaCl_2 \cdot 2H_2O$	1 ml	0·725	−0·065
0·1 N-$BaCl_2 \cdot 2H_2O$	1 ml	0·699	−0·039
0·1 N-$CoCl_2 \cdot 6H_2O$	1 ml	0·699	−0·039
0·1 N-$NiCl_2 \cdot 6H_2O$	1 ml	0·700	−0·040
0·1 N-$AlCl_3 \cdot 6H_2O$	0·2 ml	0·825	−0·165
0·1 N-$LaCl_3 \cdot 7H_2O$	0·2 ml	0·825	−0·165

* Where Δ(OD) is the optical density at 635 mμ of the dye in the absence of salt, minus the optical density at 635 mμ in the presence of salt.

metachromasy of strandin. The effect of some alcohols and acetone on the metachromasy of the strandin–TBO working solution was studied (Fig. 9). All the alcohols studied suppress the metachromasy of strandin to a variable extent. The order of suppression is:—

n-butanol > *n*-propanol > ethanol > methanol

This relationship has been noted previously by BANK and BUNGENBERG DE JONG (1939) in studying the relative effectiveness of alcohols in suppressing the metachromasy of the system sodium arabinate–toluidine blue O. The curve for *n*-butanol could not be extended further owing to phase separation of the butanol–H_2O system.

The effect of acetone is roughly comparable to that of *n*-propanol (Fig. 9).

Reaction of strandin with cationic dyes of various classes

We have studied one member of each class of metachromatic dyes whose structures and observed color shifts are shown in Table 2. The behavior of strandin with TBO has received elaborate attention in this paper, since its spectral changes are greater than

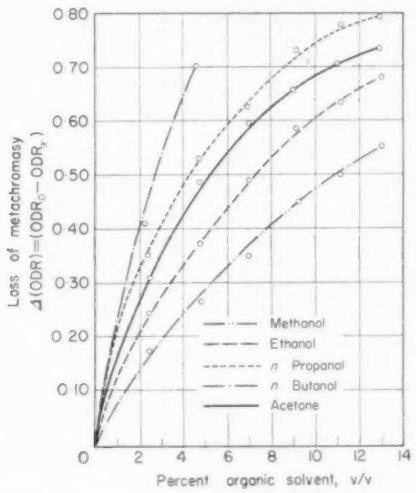


FIG. 9.—The relative effect of some organic solvents in suppressing the metachromasy of the strandin-toluidine blue O working solution.

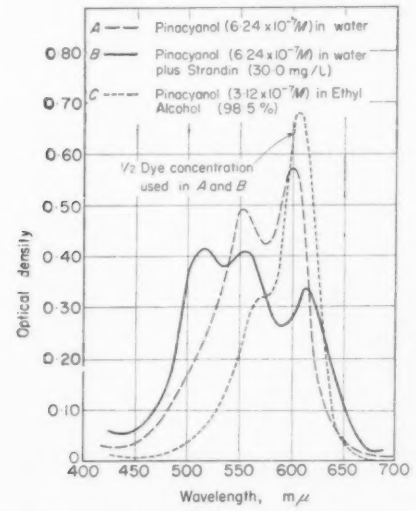
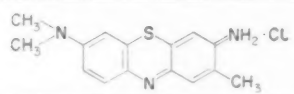
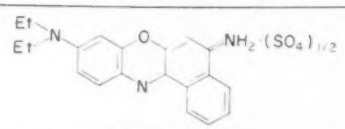
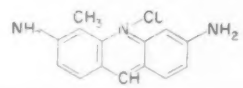
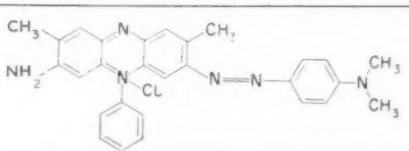
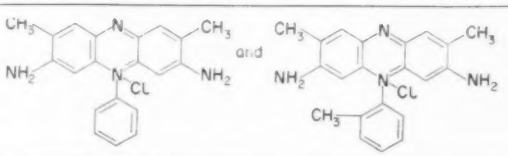
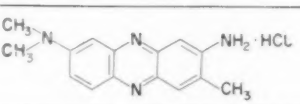
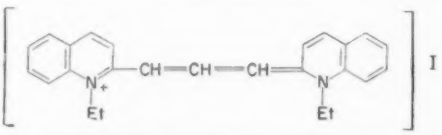


FIG. 10.—The effect of strandin on the absorption spectrum of pinacyanol.

those of any of the other dyes which we have considered. The reaction of strandin with the quinoline dye, pinacyanol, is of special interest. In water at room temperature it shows two maxima (Fig. 10), which we consider to be the α-band (600 mμ) and the β-band (550 mμ), respectively. In ethanol, the β-band reverts to a shoulder at 565 mμ

TABLE 2.—THE EFFECT OF STRANDIN ON THE
(One ml of strandin (60 mg %)
Dye concentration as indicated;

Dye class	Dye	Structure
Thiazine	Toluidine blue O	
Oxazine	Nile blue sulphate	
Xanthine	Acriflavine	
Azo	Janus green B	
Azine	Safranin O	
Azine	Neutral red	
Phenylmethane	Crystal violet	
Quinoline	Pinacyanol	

VOL.
5
1959/6

ABSORPTION SPECTRA OF SOME CATIONIC DYES
diluted to 20 ml with water)
reaction at room temperature

Spectral change		Obs. visual change		Conc. of dye
$\Delta\lambda$ (max)$\mu\mu$	Δ O.D. (max)	ortho	meta	
635 → 565 = −70	0·700 → 0·470 = −0·230	Blue	Purple (26°) Red (6°)	$1{\cdot}06 \times 10^{-5}$ M
640 → 600 = −40	0·570 → 0·442 = −0·128	Blue	Duller blue	$1{\cdot}17 \times 10^{-5}$ M
(0·2 ml of strandin 60 mg %) 445 → 447·5 = +2·5	0·580 → 0·475 = −0·105	Quenching of fluorescence		$1{\cdot}92 \times 10^{-5}$ M
610 → 580 = −30	0·640 → 0·621 = −0·019	Blue	Blue-purple	$1{\cdot}47 \times 10^{-5}$ M
520 → 510 = −10	0·700 → 0·558 = −0·142	Pink	More intense Pink	$1{\cdot}59 \times 10^{-5}$ M
475 → 510 = +35	0·640 → 0·765 = +0·125	Red-brown	Darker red-brown	$4{\cdot}26 \times 10^{-5}$ M
590 → 595 = +5	0·58 → 0·620 = +0·040	Violet	Darker violet	$6{\cdot}84 \times 10^{-6}$ M
See text		Blue	Red-purple	$6{\cdot}24 \times 10^{-7}$ M

and the α-band undergoes a slight shift to about 608 mμ. It is in the presence of strandin that we find what appear to be three bands occurring simultaneously, the α-band appearing at a wavelength of 615 mμ, the β-band at 555 mμ, and the metachromatic or γ-band at 520 mμ.

Thus, of the various dyes studied by us, five manifest positive metachromasy with strandin, their order being:—

toluidine blue O > pinacyanol > nile blue sulphate > safranin O > janus green

Two dyes show negative metachromasy (KELLY, 1956*b*), namely, neutral red and crystal violet, whereas with acriflavine there is fluorescence quenching, in addition to a spectral change.

DISCUSSION

Of the number of hypotheses regarding the nature of the change which occurs in the metachromatic reaction (HALE, 1957; KELLY, 1956*c*) the polymerization concept of MICHAELIS (1947) has received the widest acceptance. He postulated that metachromasy involves a polymerization of the dye molecules about the chromotrope, resulting in the formation of a dye–chromotrope aggregate or complex. He further suggested that the wavelength of maximal absorption is a function of the degree of polymerization of the dye, and described three characteristic absorption bands. The band characteristic of the normal (orthochromatic) form of the monomeric dye he designated as the α-band. The band, lying about 30–60 mμ below the α-band and representing the dye in the dimeric state, was indicated as the β-band. This band may also occur in the absence of the chromotrope. Finally, the γ-band which may appear between 60–100 mμ below the β-band, was indicated as representing the dye in its polymeric form.

In interpreting the results of the work presented in this paper, we shall adopt as a working hypothesis the concept of metachromasy as a manifestation of reversible polymerization (MICHAELIS, 1947, 1950). This is considered to mean the aggregation of additional dye molecules over and above that required for electrostatic binding of the anionic sites on the strandin molecule.

Thus, the loss of metachromasy attendant on an increased strandin concentration may result from a decrease in the number of dye molecules aggregated per strandin molecule. The effect of increased temperature may be explained simply as being due to the destruction of the weak bonds causing aggregation with resulting loss of metachromasy. MICHAELIS (1950) stated that the temperature effect is a reversible one. However, this was not our experience. We found that when we started from a higher temperature at which metachromasy had been lost, and recooled the system, metachromasy would recur, but not to the same degree as before. We further found that the overall colour density had diminished. Thus, we did not find the temperature phenomenon to be completely reversible.

The effects of protein and inorganic salts may be construed as indicating cationic repression. As indicated in a previous paper dealing with CSA (HARRIS and SOBEL, 1958), the mechanism may be related to the capacity of the cation to destroy the metachromatic aggregate. This is indicative of competition between cation and dye for the strandin molecule. Hydrogen ion may be considered to function as a cation. In this case, it seems probable that the result of the interaction involves suppression of the ionization of the carboxyl groups of neuraminic acid. In so far as alkali is concerned, up to a pH of about 8·5, hydroxyl ions have little or no effect on the system

VOL.
5
1959/6

(Fig. 5). It is probable that the reason lies in the unimpaired ionization of the carboxyl groups in the pH range given, i.e. 6·0–8·5.

The effect of organic solvents may be interpreted according to a mechanism proposed by RABINOWITCH and EPSTEIN (1941), i.e. as due to the interaction of dye with solvent, breaking the metachromatic dye aggregate.

The degree of metachromasy attained in a given reaction depends on the properties of both the chromotrope and the dye. In the case of strandin we are dealing with a polymeric anion, a category characteristic of most chromotropes, such as heparin, CSA, polymetaphosphate and the nucleic acids. According to SYLVÉN (1954), if the distance between neighbouring anionic groups is 5 Å or less metachromasy can occur, the degree increasing as this distance becomes less than 5 Å. The degree of metachromasy also depends on the nature of the anionic groups distributed on the chromotrope surface. The anionic groups, placed in the order of their relative capacity to produce metachromasy (HALE, 1957), are:

$$R—OSO_3^- > R—OPO_3^- > R—COO^- > R—CH_2COO^-$$

In the case of strandin it seems likely that we are dealing with carboxyl groups derived from neuraminic acid, those from the fatty acid present being bound to sphingosine in the ganglioside molecule (ZILLIKEN and WHITEHOUSE, 1958).

The degree of metachromasy will also depend on the nature of the dye involved and its capacity for aggregation. In general those dyes which show metachromasy manifest deviations from linearity in the relation between molar extinction coefficient and concentration; they do not adhere strictly to BEER's Law.

Prior to this work, and during the period in which it was completed, strandin has been assumed to be a distinct entity (FOLCH *et al.*, 1951, 1957, 1959). Results of a recent investigation by MELTZER (1958) who employed a three phase counter-current distribution technique, indicated that strandin may consist of from 8 to 15 components depending on its degree of purity. Since none of these components was obtained in pure form, and it is considered possible that they may have resulted from degradation of a pure starting material, the relationship between the apparent heterogeneity of strandin and its metachromasy poses a problem which may provide further information concerning the nature of the metachromatic reaction of strandin.

SUMMARY

1. The influence of a number of factors on the metachromasy of strandin has been studied. It has been shown that the metachromatic behavior of the solution is influenced by the concentration of strandin, the temperature, the pH, the presence of cations, and by organic solvents. Insofar as its behavior towards these factors is concerned, strandin appears to function as a typical metachromatic chromotrope.

2. Although strandin has been shown to give positive metachromasy with a number of dyes, namely, toluidine blue O, nile blue sulphate, janus green B, pinacyanol, and safranin O, we have concentrated our attention on the first of these. Neutral red and crystal violet showed negative metachromasy, whereas with acriflavine strandin caused fluorescence quenching.

3. A discussion based on the polymerization theory of MICHAELIS has been presented to account for the phenomena observed with strandin.

Acknowledgements—We are grateful to Dr. BRUNO W. VOLK, Director of Research of the Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, for his encouragement of this study. We are indebted to Drs. J. FOLCH-PI and M. LEES for introducing us to the methodology involved in the preparation and analysis of strandin. We should also like to thank Mr. DANIEL BASSUK for his technical assistance, and Mrs. LILLIAN SALOWITZ for editing and typing the manuscript.

REFERENCES

BANK O. and BUNGENBERG DE JONG, H. G. (1939) *Protoplasma* **32,** 489.

BRANTE G. (1957) In *Cerebral Lipidoses* (Edited by Van Bogaert W., CUMMINGS J. N. and LOWENTHAL A.) pp. 164–185 Thomas, Springfield, Ill.

FOLCH J., ARSOVE S. and MEATH J. A. (1951) *J. biol. Chem.* **191,** 819.

FOLCH J. and LEES M. (1959) *Amer. J. Dis. Child.* **97,** 730.

FOLCH J., LEES M. and SLOANE-STANLEY G. H. (1957) *J. biol. Chem.* **226,** 497.

HALE A. J. (1957) In *International Review of Cytology* (Edited by BOURNE G. H. and DANIELLI J. T.) Vol. VI, pp. 241–251. Academic Press, New York.

HARRIS A. F. and SOBEL A. E. (1958) *Arch. Biochem. Biophys.* **74,** 345.

KELLY J. W. (1956*a*) *The Metachromatic Reaction,* pp. 8–9. Springer-Verlag, Wien.

KELLY J. W. (1956*b*) *The Metachromatic Reaction,* p. 49. Springer-Verlag, Wien.

KELLY J. W. (1956*c*) *The Metachromatic Reaction,* pp. 68–76. Springer-Verlag, Wien.

LISON L. (1935) *Arch. biol.* **46,** 599.

MELTZER H. L. (1958) *J. biol. Chem.* **233,** 1327.

MICHAELIS L. (1947) *Cold Spr. Harb. Symp. quant. Biol.* **12,** 131.

MICHAELIS L. (1950) *J. phys. Chem.* **54,** 1.

RABINOWITCH E. and EPSTEIN L. F. (1941) *J. Amer. chem. Soc.* **63,** 69.

ROSENBERG A. and CHARGAFF E. (1959) *Amer. J. Dis. Child.* **97,** 739.

SAIFER A. and GERSTENFELD S. (1957) *J. Lab. clin. Med.* **50,** 17.

SYLVÉN B. (1954) *Quart. J. micr. Sci.* **95,** 327.

WIAME J. M. (1947) *J. Amer. chem. Soc.* **69,** 3146.

ZILLIKEN F. and WHITEHOUSE M. W. (1958) In *Advances in Carbohydrate Chemistry* (Edited by WOLFRAM M. L. and TIPSON R. S.) **13,** 242–243.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 231–235. Pergamon Press Ltd. Printed in Northern Ireland

THE DISTRIBUTION OF MUCOPOLYSACCHARIDES IN THE CYTOPLASM OF VERTEBRATE NERVE CELLS

NORMAN M. SULKIN
Department of Anatomy, Bowman Gray
School of Medicine, Winston-Salem, N.C.

(*Received* 18 *August* 1959)

THE occurrence of mucopolysaccharides in the cytoplasm of nerve cells was first demonstrated in the autonomic and sensory ganglion cells of the senile dog (SULKIN and KUNTZ, 1952). With the periodic acid-Schiff method this material was observed mainly in the form of a non-granular cytoplasmic deposition in autonomic ganglion cells. In a more recent study (SULKIN, 1955*a*) the mucopolysaccharide was also observed in the form of discrete granules which could be distinguished from PAS positive pigment granules by a variety of histochemical tests. Moreover, this PAS positive mucopolysaccharide was limited in its distribution to the peripheral ganglion cells of the senile dog, and it was suggested that the granular form represented a stage in the accumulation of mucopolysaccharide during the process of aging.

With the development of newer histochemical methods for demonstrating and studying mucopolysaccharides (KRAMER and WINDRUM, 1953, 1954; SCOTT and CLAYTON, 1953) the distribution of this substance was again studied in the nerve cells of the dog (SULKIN, 1955*b*). The method of KRAMER and WINDRUM is based on the sulphation of neutral mucopolysaccharides in tissue sections which are then visualized by their metachromatic staining properties following toluidine blue staining. When applied to peripheral ganglia in the senile dog, this resulted in preparations which were comparable to the periodic acid-Schiff (PAS) method. In those instances in which the mucopolysaccharide appeared as a non-granular cytoplasmic deposition by use of the PAS method, it also appeared as a metachromatic non-granular deposition following the sulphation–toluidine blue technique. In the instances in which the mucopolysaccharide appeared as positive granules following the PAS technique they appeared as large metachromatic granules following the sulphation method. Moreover, with the use of the latter method, it was demonstrated that small metachromatic granules were present in the nerve cells in the central nervous system as well as in peripheral nerves and in dogs of all ages as well as in the senile animals. A large series of histochemical tests indicated that these smaller metachromatic granules were identical in nature to the large mucopolysaccharide granules and the mucopolysaccharide non-granular cytoplasmic deposition that characterized the peripheral neurons of the senile dog. Studies conducted with the use of GOMORI's (1950) aldehyde–fuchsin stain following periodic acid oxidation as well as sulphation with concentrated sulphuric acid resulted in similar observations. On the basis of these studies it was concluded

* There is some evidence (SULKIN, 1955*a*) that the polysaccharides with whose presence in nerve cells this paper deals are combined with proteins, but since this has not been established with certainty, the term, mucopolysaccharide, instead of mucoprotein, will be used.

that nerve cells in the dog contain mucopolysaccharides which are present in insufficient quantities to be demonstrated by the PAS method except in peripheral ganglia of senile animals.

The present study was undertaken to determine the occurrence and distribution of mucopolysaccharides in the cytoplasm of nerve cells in a variety of vertebrate species including man.

MATERIALS AND METHODS

The materials used in this study included specimens of cerebrum, cerebellum, spinal cord, autonomic ganglia and sensory ganglia from a variety of vertebrate species (Table 1).

TABLE 1.—A LIST OF VERTEBRATES, INCULDING THE TISSUES FROM EACH SPECIES WHICH WERE STUDIED, ARRANGED IN TABULAR FORM

	Autonomic ganglia	Sensory ganglia	Spinal cord	Cerebral cortex	Cerebellum
Man	X		X		
Monkey *Rhesus maccus mullata*	X	X			
Dog	X	X	X	X	X
Sheep			X	X	X
Cat	X	X			
Guinea Pig	X	X	X	X	X
Rat	X	X	X	X	X
Mouse	X			X	X
Chicken			X	X	
Frog *Rana pipiens*			X	X	
Ambystoma trigonium				X	
Amphioxus lanceolata				X*	
Necturus				X	
Squalus acanthius			X	X	X

* Cerebral vesicle

The specimens were fixed in 10% formalin, dehydrated with alcohol, cleared with xylol and embedded in paraffin in the usual manner. Glycogen was removed from the sections by placing them in a 37° incubator for one hr in a 1% solution of diastase prepared according to LILLIE (1948).

The Schiff reaction was performed on sections following oxidation with periodic acid. Other sections were oxidized with peracetic and performic acids prepared by the methods described by

VOL. 5 1959/6

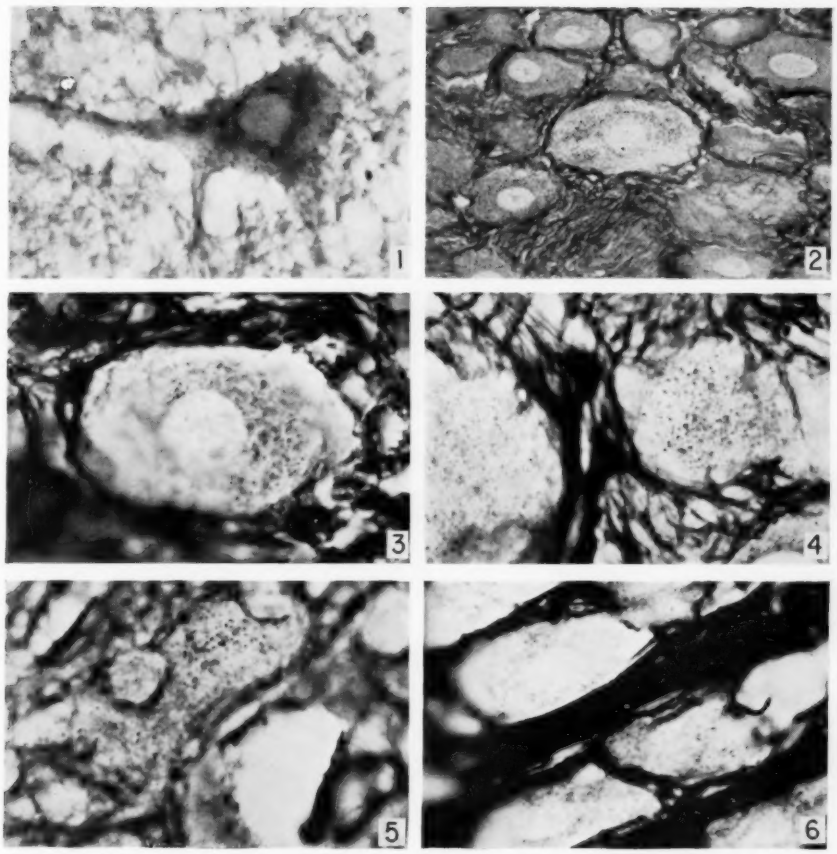


FIG. 1.—Photomicrograph of ventral horn cell from a section of the spinal cord of a sheep showing PAS positive mucopolysaccharide granules. 10× ocular; 44× objective.

FIG. 2.—Photomicrograph of autonomic ganglion cell from the superior cervical ganglion of a two month old cat showing PAS positive mucopolysaccharide granules. 10× ocular; 44× objective.

FIG. 3.—Photomicrograph of sensory ganglion cells from a section of a spinal ganglion of a guinea pig showing metachromatic granules following the sulphation–toluidine blue technique. 10× ocular; 44× objective.

FIG. 4.—Photomicrograph of autonomic ganglion cells from a section of a superior cervical ganglion of a guinea pig showing metachromatic granules following the sulphation–toluidine blue technique. 10× ocular; 44× objective.

FIG. 5.—Photomicrograph of ventral horn cell from a section of the spinal cord of a guinea pig showing metachromatic granules following the sulphation–toluidine blue technique. 10× ocular; 44× objective.

FIG. 6.—Photomicrograph of autonomic ganglion cells from a section of the superior cervical ganglion of a two month old cat showing metachromatic granules following the sulphation–toluidine blue technique. 10× ocular; 44× objective.

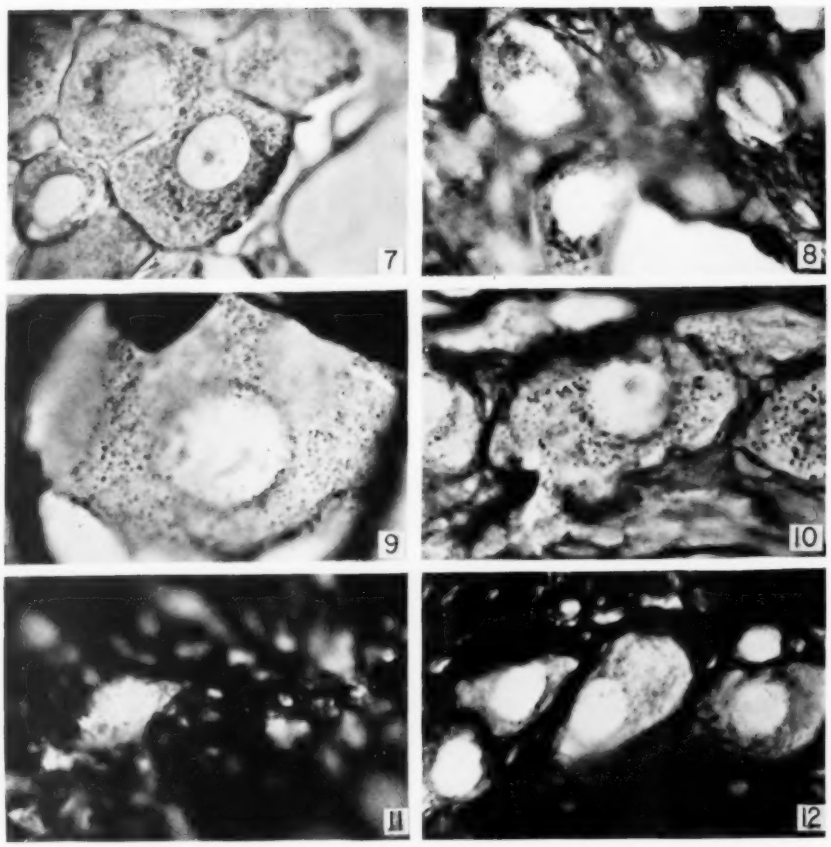


FIG. 7.—Photomicrograph of sensory ganglion cells from a section of a spinal ganglion from a young rat showing metachromatic granules following the sulphation–toluidine blue technique. 10× ocular; 44× objective.

FIG. 8.—Photomicrograph of autonomic ganglion cells from a section of the superior cervical ganglion of a young rat showing metachromatic granules following the sulphation–toluidine blue technique. 10× ocular; 44× objective.

FIG. 9.—Photomicrograph of sensory ganglion cells from a section of a spinal ganglion of a young dog showing metachromatic granules following the sulphation–toluidine blue technique. 10× ocular; 44× objective.

FIG. 10.—Photomicrograph of autonomic ganglion cells from the superior cervical ganglion of a young dog showing metachromatic granules following the sulphation–toluidine blue technique. 10× ocular; 44× objective.

FIG. 11.—Photomicrograph of a dorsal horn cell from a section of the spinal cord of a chick showing the metachromatic granules following the sulphation–toluidine blue technique. 10× ocular; 44× objective.

FIG. 12.—Photomicrograph of an autonomic ganglion cell from the section of a thoracic sympathetic ganglion of a human foetus showing the metachromatic granules following the sulphation–toluidine blue technique. 10× ocular; 44× objective.

VOL.
5
1959/6

LILLIE (1954) and PEARSE (1953). This was one of the methods used for distinguishing mucoprotein from lipofuchsin pigment.

Sulphation of tissues was performed on deparaffinized sections, the methods of KRAMER and WINDRUM (1953, 1954) being followed, in general, as described earlier (SULKIN, 1955*b*). The length of time necessary for sulphation varied from 2 to 6 min depending upon the species.

Aldehyde–fuchsin stain was prepared according to the method described by GOMORI (1950). Sections were stained in the aldehyde–fuchsin mixture from 5 to 45 min after oxidation with periodic acid, sulphation with concentrated sulphuric acid, or no prior treatment. In all instances glycogen was digested with diastase.

Further to distinguish between lipoidal pigments and mucopolysaccharide granules, acetylation of the sections was accomplished by placing them in a mixture containing 40 parts acetic anhydride and 60 parts pyridine for 24 hr at room temperature. Deacetylation was accomplished by treating acetylated sections with 0·1 N-KOH for 40 min according to the method of MCMANUS and CASON (1950).

The oxidation potential of the PAS-positive materials and metachromatic materials was studied by LILLIE's (1954) modification of SCHMORL's (1928) ferric–ferricyanide reduction test. Sections prepared for the demonstration of acid fastness in these materials were stained with carbolfuchsin overnight at room temperature and decolorized with 3% HCl in 70% alcohol for intervals varying from 3 min to 4 hr.

OBSERVATIONS

Except for those species in which the ages of the animals were unknown (A. trigonium, Amphioxus, Necturius and Squalus) the studies were confined to young animals so that the observations would not be obscured by the presence of lipofuchsin pigment. Human nerve tissue was obtained from individuals under 17 years of age and included specimens from immature foetuses. No attempt was made at comparative quantitive studies amongst the different species.

A study of the various nerve cells from all the species following the periodic acid-Schiff reaction indicated that isolated cells in different parts of the nervous system of the monkey, guinea pig, rat, mouse, and frog contained very small and widely scattered PAS positive granules which could be identified as mucopolysaccharides (Fig. 1). In the cat, however, including 3 kittens under 2 months of age, the autonomic and sensory ganglion cells were characterized by a heavy concentration of PAS positive granules in the cytoplasm (Fig. 2). The histochemical procedures which were employed all indicated that these granules were mucopolysaccharide in nature.

These tests included a negative reaction of the granules when stained with Sudan black, a negative reaction to the acid-fast stain, an inability to reduce in SCHMORL's ferric–ferricyanide mixture, a negative reaction to the Schiff reagent following oxidation with performic and peracetic acids, and the negative reaction resulting from acetylation prior to treatment with the Schiff reagent. Treatment of the acetylated sections with dilute KOH prior to staining with the Schiff reagent brought back a positive reaction.

Observation of sections which were sulphated and stained with toluidine blue revealed that metachromatic granules were present in all the nerve cells of all the species which were studied (Figs. 3–12). These metachromatic granules were identical in their appearance, distribution and chemical reactions to the mucopolysaccharide granules which have previously been described in the nerve cells of the dog (SULKIN, 1955*b*).

It has been reported by GOMORI (1950) and SCOTT and CLAYTON (1953) that aldehyde–fuchsin is able to combine with aldehydes to yield purplish dyes that have staining properties different from the fuchsin itself. Sections of nervous tissue from

the various species oxidized with periodic acid and treated with the aldehyde–fuchsin stain were characterized by a staining reaction parallel to that which resulted from the use of the sulphation–toluidine blue procedure.

Sections of nerve tissue stained with the aldehyde–fuchsin stain following sulphation also gave results which paralleled those of sections treated with the sulphation–toluidine blue technique. Sections which were stained with aldehyde–fuchsin without prior oxidation or sulphation gave a negative reaction.

DISCUSSION

The data obtained in this study are an extension of previously reported observations on the nerve cells of the dog. It is felt that a sufficient number of vertebrate species have been investigated to warrant the conclusion that the presence of metachromatic granules following sulphation and toluidine blue staining is a universal feature of vertebrate nerve cells. The conclusion that these metachromatic granules represent mucopolysaccharides is based on evidence which has been previously reported for homologous granules in the nerve cells of the dog (SULKIN, 1955*b*) and from the results reported by KRAMER and WINDRUM (1953). The latter have offered evidence that the treatment of tissue sections with concentrated sulphuric acid results in esterification of carbohydrates which in turn is responsible for the subsequent metachromasia. Their studies on tissue sections of a large variety of tissues and organs present data which indicate the close parallelism between the distribution of PAS-positive material and the metachromatic substances produced by sulphation. The evidence obtained by KRAMER and WINDRUM was further supported by SULKIN (1955*b*) who demonstrated the parallel distribution, staining and histochemical reactions of the metachromatic substance following sulphation. It further demonstrated the PAS positive mucopolysaccharides in the ganglion cells of the senile dog, in which there is a heavy accumulation of PAS positive mucopolysaccharide in the cytoplasm as well as in the basement membranes and in the brush borders of the proximal convoluted tubules of the kidney. The data obtained in this study indicated, moreover, that the sulphation–toluidine blue procedure is a more sensitive method for the detection of mucopolysaccharides than is the PAS reaction.

On the basis of these findings it is suggested that the cytoplasm of nerve cells in vertebrates is characterized by the presence of mucopolysaccharide which, except in the cells of the senile dog and in some instances in young animals of other species, is not concentrated enough to be demonstrated by the PAS method.

The reactions of aldehyde–fuchsin and the Schiff reagent following periodic acid oxidation have been compared by SCOTT and CLAYTON (1953). Their observations indicate that these substances act in much the same way except in beta cell granules in which the aldehyde–fuchsin gives a strong reaction and the Schiff reagent is negative, and in reticular tissue and Paneth cell granules in which the Schiff reagent gives a stronger reaction than the aldehyde–fuchsin stain. Furthermore, according to SCOTT and CLAYTON, aldehyde–fuchsin without prior oxidation stains certain mucopolysaccharides including those which contain a high percentage of chondriotin sulphuric acid. They conclude, therefore, that aldehyde–fuchsin may have an affinity toward sulphate groups.

If tissues containing mucopolysaccharides are sulphated with concentrated sulphuric acid to cause the esterification of these carbohydrate moieties, it would be

VOL.
5
1959/6

expected that such sections would reveal a positive staining reaction with the aldehyde–fuchsin stain, if it is assumed that aldehyde–fuchsin does have an affinity for sulphated mucopolysaccharides. The data obtained in a previous study (SULKIN, 1955*b*) together with the present results, support the view that aldehyde–fuchsin does react in a positive manner to the granules following sulphation of the sections and present further evidence for the occurrence of mucopolysaccharides in the cytoplasm of nerve cells.

The role of mucopolysaccharides in the cytoplasm of nerve cells has not yet been established. In a series of reports, HESS (1953, 1955*a*, 1955*b*) has concluded that the ground substance of the central nervous system contributes to the constitution of the blood-brain barrier and is the substance that causes normal adult brains to resist vital staining by intravenous or intraperitoneal trypan blue. This ground substance consists of a mucoprotein which, the author suggests, is elaborated and maintained by nerve cells and neuroglia. The finding of mucopolysaccharides, which may be present in mucoproteins (SULKIN, 1955*a*), in the cytoplasm of nerve cells is in accord with the suggestion of HESS.

SUMMARY

A systematic study of nerve cells from various parts of the nervous system of 16 vertebrate species from amphioxus to man provides data which indicate that the cytoplasm of nerve cells in various portions of the nervous system in each of the species is characterized by the presence of mucopolysaccharides. The mucopolysaccharides appear in the form of small granules which in some instances are revealed with the use of the PAS techique, but which in most instances are revealed only following sulphation. The sulphation changes the mucopolysaccharide to acid conjugated mucopolysaccharides which can then be demonstrated by the metachromatic colouring reaction following toluidine blue staining. In the nerve cell of the senile dog, the mucopolysaccharides are apparently sufficiently concentrated in the cytoplasm so that they form a non-granular mass which can be demonstrated by the PAS technique as well as by the sulphation–toluidine blue method. The methods of distinguishing the mucopolysaccharides from lipofuchsin pigment are described.

REFERENCES

GOMORI G. (1950) *Amer. J. clin. Path.* **20,** 665.
HESS A. (1953) *J. comp. Neurol.* **98,** 69.
HESS A. (1955*a*) *J. comp. Neurol.* **102,** 65.
HESS A. (1955*b*) *Arch. Neurol. Psyschiat., Chicago* **73,** 380.
KRAMER H. and WINDRUM G. M. (1953) *J. clin. Path.* **6,** 239.
KRAMER H. and WINDRUM G. M. (1954) *J. Histochem. Cytochem.* **2,** 196.
LILLIE R. D. (1948) *Histopathologic Technic.* Blakiston, Philadelphia, Pa.
LILLIE R. D. (1954) *Histopathologic Technic and Practical Histochemistry.* Blakiston, Philadelphia, Pa.
MCMANUS J. F. A. and CASON E. J. (1950) *J. exp. Med.* **91,** 651.
PEARSE A. G. E. (1953) *Histochemistry, Theoretical and Applied.* Little, Brown, Boston, Mass.
SCOTT H. R. and CLAYTON B. P. (1953) *J. Histochem. Cytochem.* **1,** 336.
SCHMORL G. (1928) *Die Pathologisch—histologischen Untersuchungmethoden.* Vogel, Leipzig.
SULKIN N. M. (1955*a*) *J. Geront.* **10,** 135.
SULKIN N. M. (1955*b*) *J. biophys. biochem. Cytol.* **1,** 459.
SULKIN N. M. and KUNTZ A. (1952) *J. Geront.* **7,** 533.

Journal of Neurochemistry, 1960, Vol. 5, p. 236-244. Pergamon Press Ltd. Printed in Northern Ireland

DEOXYRIBONUCLEIC ACIDS OF CENTRAL NERVOUS SYSTEM, KIDNEY, AND SPLEEN: A COMPARISON OF SOME CHEMICAL AND PHYSICAL PROPERTIES*

C. F. EMANUEL and I. L. CHAIKOFF
Department of Physiology, University of California, Berkeley

(*Received* 21 *September* 1959)

VOL.
5
1959/6

THE neurone of the mature brain is incapable of undergoing mitosis, and it is therefore not surprising that processes dependent on mitotic acitivity, namely, regeneration and neoplasia, occur rarely, if at all, in that cell. Since deoxyribonucleic acid (DNA) synthesis precedes cell division (BLOCH, 1958), we became interested in the possibility that mitosis is blocked in the neurone by some structural peculiarity of its DNA. For this reason we have compared some physical and chemical properties of DNA isolated from brain with those of DNA isolated from kidney and spleen, two tissues that show mitotic activity.

Few attempts to isolate fibrous DNA from the central nervous system have been reported (BENDICH *et al.*, 1956), probably because of the unfavourable ratio between nuclear and non-nuclear material in this system. Recently, we (EMANUEL and CHAIKOFF, 1957*b*) described a method for isolating fibrous DNA from tissues with low DNA content, based on actual removal of nuclei from these tissues by controlled homogenization and subsequent collection of the nuclei by adsorption. A modification of this method was used to prepare fibrous DNA from the cerebellum.

EXPERIMENTAL

Isolation of DNA. Cattle spleen and kidney DNA were isolated by our earlier method (EMANUEL and CHAIKOFF, 1957*b*). In a modified form, the same procedure is well adapted for nervous tissue, since it facilitates the removal of the large amounts of non-nuclear material characteristic of this tissue. Cerebellum DNA containing a minimal amount of protein and ribonucleic acid was prepared as follows: 100 g of cerebellum cortex, obtained from cattle about 20 min after they had been sacrificed, were homogenized in two equal batches, each with 25 ml of arsenate buffer (EMANUEL and CHAIKOFF, 1957*a*). The 38 μ orifice in the hydraulic homogenizer was used. 175 ml of arsenate buffer were added to the pooled homogenates and the well-mixed suspension was further homogenized (EMANUEL and CHAIKOFF, 1957*a*, 257–8) continuously through the 25 μ orifice. The product obtained was washed through nylon cloth with 310 ml of buffer. The filtrate was first well mixed with 42 g of Hyflo-Super Cel (diatomaceous earth) with the aid of a Teflon mixing paddle and was filtered in at least six small batches through an 11 cm Büchner funnel.† A Whatman No. 1 filter paper overlaid with 2–3 mm of Hyflo-Super Cel was used for each filtration. Each filter pad containing the adsorbed nuclei was washed with two 50 ml portions of chilled buffer. The combined filter pads were suspended in 100 ml of buffer and the resulting slurry was saturated with solid NaBr. The suspension was well mixed until it became free flowing. It was filtered in six small batches and the combined filtrates were poured into 3 volumes of ethanol. The DNA so precipitated was removed with a

* Aided by a grant from the United Cerebral Palsy Research and Educational Foundation.

† All isolation operations were carried out in jacketed vessels maintained at a temperature of 0° with circulating brine.

glass rod and redissolved in 60 ml of the arsenate buffer. Ten g of the Hyflo-Super Cel were added to the DNA solution and the resulting mixture was again filtered. The viscous, colorless solution was centrifuged for one hr at 100,000 **g**. The DNA in the supernatant fractions was again precipitated with ethanol and redissolved in arsenate buffer by continuous slow mixing at 0°, and the resulting solution was saturated with solid NaBr. After the addition of Hyflo-Super Cel, the suspension was filtered, and the DNA in the filtrate was precipitated a third time as described above. The fibrous DNA, when dissolved in 20 ml of 1/4th strength arsenate buffer and filtered (through Hyflo-Super Cel), was ready for study and analysis. The DNA solution was stored on dry ice until used. The yields of DNA from 12 separate preparations ranged from 45 to 80 mg per 100 g of fresh tissue—yields about 1/3 those obtainable from a tissue such as liver. The steps in the isolation of the cerebellum DNA are these:

100 g fresh cattle cerebellum cortex
↓
Homogenized with 50 ml of buffer (38 μ orifice)
↓
Rehomogenized with 175 ml buffer (25 μ orifice)
↓
Strained through nylon cloth with 310 ml buffer ——→ Connective tissue discarded
↓
Filtrate mixed with 42 g Hyflo-Super Cel and filtered in 6 small batches. Filter pads well washed with buffer ——→ Filtrates discarded
↓
Filter pads suspended in 100 ml of buffer and slurry saturated by addition of excess solid NaBr
↓
Filtered in 6 small batches ——→ Filter pads discarded
↓
Combined filtrates mixed with 3 volumes ethanol ——→ Liquid discarded
↓
Precipitated DNA redissolved in 60 ml buffer and filtered through Hyflo-Super Cel
↓
Filtrate centrifuged 1 hr (100,000 **g**) and supernatant fractions precipitated with ethanol ——→ Liquid discarded
↓
Precipitated DNA redissolved in 50 ml buffer, solution saturated by addition of solid NaBr. Solution filtered through Hyflo-Super Cel and DNA precipitated with ethanol ——→ Liquid discarded
↓
Precipitated DNA redissolved in 1/4 strength buffer and filtered through Hyflo-Super Cel. DNA solution stored on dry ice.

Physical measurements were carried out on DNA samples that had not been dialysed, dried, or exposed to ether or ethanol concentrations in excess of 75 per cent—procedures known to inactivate nucleic acids biologically (ZAMENHOF, 1956).

Analytical procedures. The methods used for nitrogen and phosphorus analyses have been described elsewhere (EMANUEL and CHAIKOFF, 1957*b*). Ascending paper chromatography of DNA hydrolysates was carried out with *iso*propyl alcohol as solvent (WYATT, 1951). DNA concentrations were determined by the Stumpf reaction (STUMPF, 1947).

For *spectrophotometry* we used a calibrated Beckman DU instrument which had been corrected for parallax (EMANUEL and CHAIKOFF, 1958*a*); no temperature regulation was used.

Viscosity determinations were made in an Ostwald viscometer with a water delivery time of 66·4 sec. All measurements were determined at 30° ± 0·13 in 0·02 M-arsenate buffer (EMANUEL and CHAIKOFF, 1957*b*).

Optical rotations were measured at ambient temperatures (25°–27°) in a Zeiss-Winkel polarimeter (reading to 0·01°). Two decimeter micro tubes were used in conjunction with the sodium doublet

illumination. Rotation measurements were carried out in 0·08 M-arsenate buffer, pH 7·0; a few measurements were also made in 0·08 M-arsenate buffer saturated with sodium bromide (~7 M solution).

RESULTS

Chemistry

Contamination with protein. Adulterating protein was looked for by hydrolysing 10 mg of each DNA sample for 30 min at 100° with 200 μl of 4·5 N-perchloric acid in a stoppered tube. 20 μl aliquots of the hydrolysates were chromatographed and the well-dried (at room temperature) chromatograms were sprayed with buffered ninhydrin

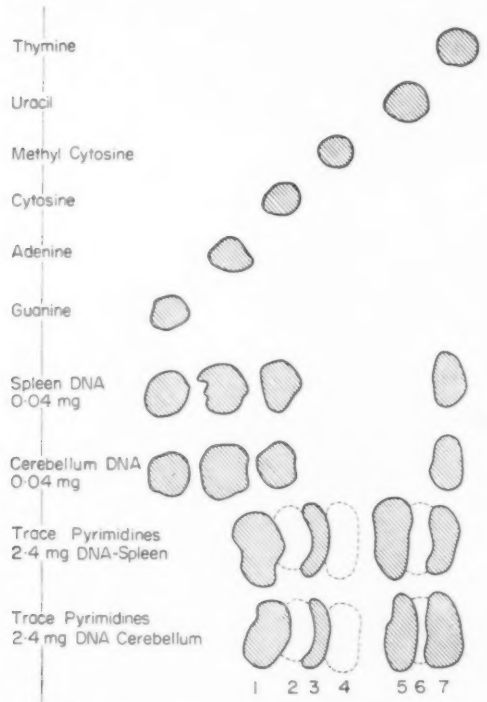


FIG. 1.—Chromatogram of spleen and cerebellum DNA of perchloric acid hydrolysates as viewed under ultraviolet lamp.
Trace pyrimidines: compound 1, green fluorescent spot; compound 2, unknown absorbing spot; compound 3, cytosine; compound 4, 5-methyl cytosine; compound 5, uracil; compound 6, unknown spot; compound 7, thymine.

(FEIGL, 1954). Ninhydrin-positive materials were not detected in spleen and cerebellum DNA by this procedure which, in a previous study (EMANUEL and CHAIKOFF, 1958*b*). was shown to be sensitive to a 1–2 per cent level of contamination.

Contamination by ribonucleic acid (RNA). To detect contaminating RNA, 4·2 mg of DNA were hydrolysed with 85 μl of 10 N-perchloric acid for 1 hr at 100° in a stoppered tube. The charred contents were centrifuged and the purines in the supernatant fluid were removed with silver perchlorate at pH 2·0 as previously described (EMANUEL and CHAIKOFF, 1958*a*). The pH of the supernatant fluid was adjusted to 6·5 with dilute base and the quantitatively precipitated pyrimidines were removed by

VOL.
5
1959/6

centrifugation. The silver was removed from the washed precipitate with HCl and filtration. The resulting filtrate containing the pyrimidines was concentrated to about 0·5 ml by directing a stream of air on its surface (pervolatilization) and the concentrate was chromatographed on washed No. 598 Schleicher and Schuell paper. Uracil and methylcytosine were not visible under ultraviolet light. The entire area between thymine near the front and cytosine near the centre (Fig. 1) was cut from the paper and eluted with warm water, and the solution obtained was concentrated to about 0·1 ml by pervolatilization. The entire concentrate was applied in 10 μl aliquots to one spot on a Whatman No. 1 paper and chromatographed. In DNA samples isolated from cerebellum and spleen, in addition to small amounts of thymine and cytosine which were inadvertently included, trace amounts of 5-methylcytosine and uracil were evident under ultraviolet illumination (Fig. 1). Also, a considerable amount of an intensely green fluorescent substance at R_F 0·43 was present in both DNA samples. The amount of uracil detected by these means would be equivalent to an RNA contamination of 1–2 per cent for both splenic and cerebellar DNA.

Composition. The purine-pyrimidine determinations of spleen and cerebellum DNA (Table 1) were carried out by an earlier described differential procedure (EMANUEL and CHAIKOFF, 1958*a*) devised for fish sperm DNA. In contrast to our data on sperm DNA, the values for the ratios of adenine to thymine and guanine to cytosine were not as close to unity as expected. However, the ratio for total purine to total pyrimidine is nearly unity, and this leads us to believe that the analytical procedures are not seriously in error. The slightly high total base yields (103–105 per cent as judged by phosphorus determinations) are probably due to extraneous absorption and stray light from trace substances shown in Fig. 1. Failure to obtain a value of unity for ratios of adenine to thymine and guanine to cytosine in the presence of equivalent amounts of total purines and pyrimidines has been repeatedly demonstrated by BENDICH *et al.* (1958) in their DNA fractionation studies.

Identity of the carbohydrate moeity of the nucleic acid samples with deoxyribose was shown by typical absorption spectra obtained by the highly specific Stumpf reaction (DISCHE, 1955).

Extent of denaturation. The degree of denaturation was tested by SHACK's spectrophotometric method (SHACK, 1958) which requires the measurement of total absorption of a DNA sample dissolved in 0·01 M-NaCl in the presence and absence of 0·01 M-Mg^{++}. The difference in absorbancy between these two measurements was equal to or less than the instability of our spectrophotometer.

Physical Properties

Spectrophotometry. There appeared to be no striking differences between ultraviolet absorption spectra of DNA samples isolated from kidney and cerebellum cortex (Figs. 2 and 3), in spectral changes produced by variable ionic strength. As was to be expected, the wavelengths of maximal and minimal absorption were 259–260 mμ and 230 mμ respectively. Essentially identical absorption curves were obtained with all samples dissolved in all concentrations of KCl between 1 M and 0·001 M. The well-known (BLOUT and ASADOURIAN, 1954; THOMAS, 1954) hyperchromic effect observed in pure water solutions was of nearly equal magnitude (16–17 per cent for cerebellum DNA and 15 per cent for kidney DNA).

To our knowledge, it has not been previously reported that a hyperchromic effect

TABLE 1.—BASE COMPOSITION OF CATTLE SPLEEN AND CEREBELLUM DNA

Compound	Spleen DNA	Cerebellum DNA
	Moles per cent	Moles per cent
Cytosine	20·2	20·1
Thymine	30·3	30·0
Adenine	28·1	27·2
Guanine	21·5	22·8
Purine-pyrimidine ratios		
$\frac{\text{Adenine}}{\text{Thymine}}$	0·93	0·91
$\frac{\text{Guanine}}{\text{Cytosine}}$	1·07	1·13
$\frac{\text{Purines}}{\text{Pyrimidines}}$	0·98	1·00
Recoveries		
Summed recovery (moles $\times 10^{-5}$)	2·40 (103%)	2·63 (105%)
Measured P recovery (moles $\times 10^{-5}$)	2·33	2·51
$\frac{\text{Gram atoms N present*}}{\text{Gram atoms P present}}$	3·89	3·88

* Corrected to 100 per cent recovery. For a tetranucleotide containing one mole each of cytosine, thymine, adenine, and guanine the ratio, $\frac{\text{gram atoms N}}{\text{gram atoms P}}$ is 3.75.

is obtained when DNA is dissolved in very high salt concentrations. Thus, when DNA is dissolved in 7 M-NaBr (saturated), the hyperchromic effect is 60 per cent as great as that observed in distilled water. Cerebellum and kidney samples showed similar high salt hyperchromicity. A detailed investigation of this new phenomenon will be reported later.†

Viscosity. The viscosity numbers (η sp/$_c$ in g/dl) of DNA samples isolated from spleen, kidney, and cerebellum are presented in Fig. 4. Because these determinations were made at moderately high shear gradients, no absolute interpretations concerning the physical state of these DNA samples can be made. However, we have repeatedly observed that the values for cerebellum DNA extrapolated to zero concentration are nearly double those for kidney and spleen nucleic acids. The significance of these differences is not clear. One is tempted to explain them on the basis of partial enzymic

† According to ZAMENHOF (personal communication) saturated NaCl solutions (5·5 M) do not diminish biological activity of the transforming factor.

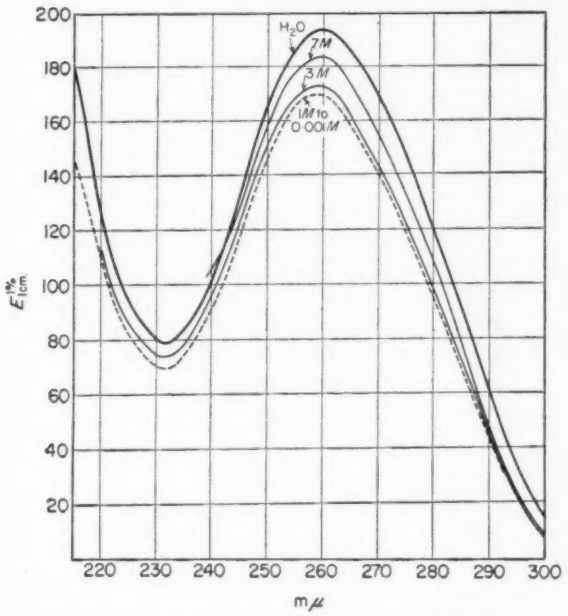


FIG. 2. Ultraviolet absorption spectra of cattle kidney DNA.

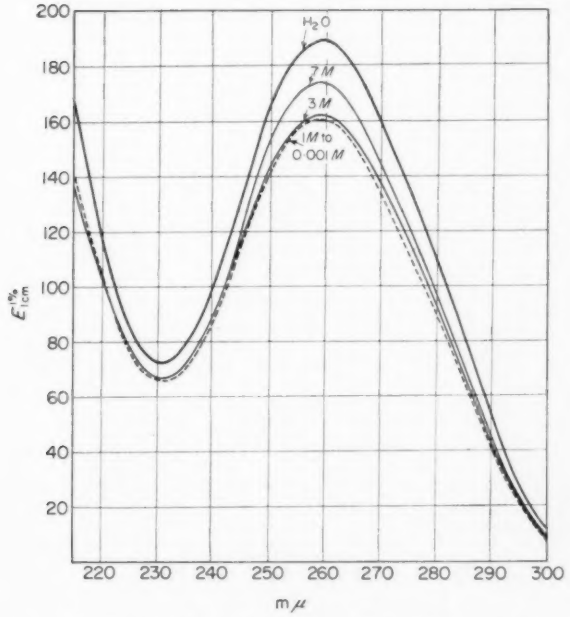


FIG. 3. Ultraviolet absorption spectra of cattle cerebellum DNA.

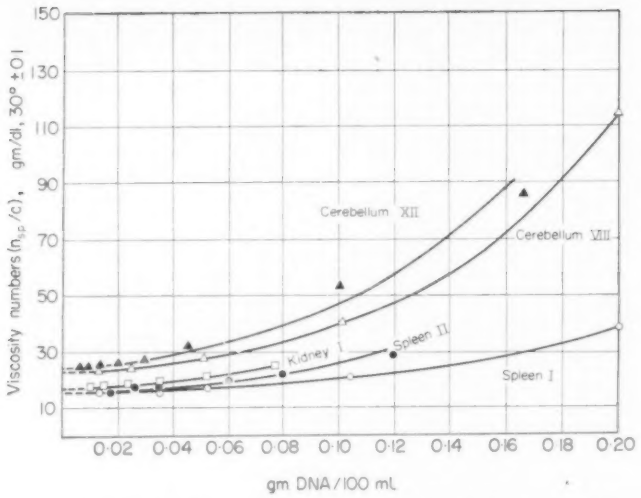


FIG. 4. Reduced specific viscosity of cattle DNA (Potassium salt).
Roman numerals refer to the separate nucleic acid preparations examined.

degradation of the spleen and kidney samples, but the close similarity of the extrapolated spleen and kidney viscosity numbers makes this explanation improbable.

Optical rotation. The specific optical rotation of nucleic acids results from the summation of at least two different effects—the helical superstructure and the carbohydrate molecular structure. The rotation values are also influenced by hydrogen bonding and by environmental conditions, such as ionic strength and pH. The data (Table 2) reveal no significant differences between the rotations of spleen and cerebellum DNA.

TABLE 2.—SPECIFIC OPTICAL ROTATIONS OF DEOXYRIBONUCLEIC ACID

Sample	In 0·08 M-arsenate buffer	In 7 M-NaBr*
Spleen DNA	$[\alpha]_D^{25-27°} = +118° \pm 10°$ (5)†	$+67{\cdot}1 \pm 3°$ (2)
Cerebellum DNA	$[\alpha]_D^{25-27°} = +116° \pm 14°$ (3)	$+71{\cdot}1 \pm 8°$ (4)

* 0·08 M-arsenate buffer saturated with NaBr.
† Figures in parentheses indicate number of separate preparations examined.

The values obtained are not unlike those reported for calf thymus DNA by TAMM *et al.* (1952).

The usual specific optical rotations of about 117° (observed in 0·1 M neutral salt solutions) were considerably depressed (Table 2) when measured in saturated NaBr solutions. This depression suggests that the DNA molecule has either lengthened or partially collapsed into a disoriented structure. The ultraviolet hyperchromicity in 7 M-NaBr (see above) lends support to this suggestion. It is, however, difficult to envisage how a high salt concentration can bring about an expansion of the helical structure, and such an expansion is still more unlikely when it is recalled that moderate neutral salt concentrations are suspected of compressing the DNA helix by shielding the charged internucleotide phosphate groups (LAWLEY, 1956). A third possibility,

VOL. 5 1959/6

namely, that the two-stranded DNA molecule has been separated into single strands by a high salt concentration, can be ruled out, since the hyperchromic effect and the optical rotation depression are reversible phenomena.

A DNA solution in saturated NaBr undergoes irreversible changes if it is heated to 100° or if it is allowed to stand for long periods at room temperature. Eventually, even at room temperature, DNA dissolved in 7 M-NaBr often precipitates in a flocculent form.

DISCUSSION

From the results presented here, it cannot be concluded unequivocally that cerebellum DNA is identical in all respects with DNA isolated from kidney and spleen. However, the differences observed between DNA of the central nervous system and DNA of the other two tissues are of quantitative rather than qualitative significance. Thus, the usual purine and pyrimidine bases, as well as deoxyribose, were found in cerebellum DNA. Furthermore, the elementary compositions were of the expected magnitudes in the DNA isolated from all three tissues. Their ultraviolet absorption spectra and hyperchromic shifts, observed when DNA is dissolved in water, in 0·1 M neutral salt or in 7 M neutral salt, were of similar form and intensity. The optical rotation values for DNA of these tissues, as determined in dilute buffer, were closely similar to those reported by TAMM *et al.* (1952).

These findings on chemical constitution, helical structure (optical rotation), and hydrogen bonding (hyperchromic shift) suggest a basic similarity in the make-up of DNA isolated from neurone and non-neurone sources. It would appear that the cell constancy of mature nervous tissue is not determined by the chemical constitution of its DNA.

The viscosity of cerebellum DNA was nearly twice that of DNA obtained from spleen or kidney. We cannot decide whether this disparity between the two indicates that DNA in the living cerebellum differs from DNA in the living kidney and spleen or whether it reflects a limited enzymic degradation that occurred during isolation of the DNA samples from the non-neurone sources. Because our viscosity data for spleen and kidney DNA are so much alike, we are inclined to believe that there does exist an inherent difference, as regards molecular weight and asymmetry, between DNA of cerebellum and that of the other two tissues. Until the anomalously higher viscosity of cerebellum DNA is further explored, it cannot be decided with certainty whether or not brain DNA differs in some physical aspects from that in other tissues. Further studies on viscosity with the rotating cylinder viscometer in conjunction with molecular weight determinations are indicated.

In our previous publication (EMANUEL and CHAIKOFF, 1957*a*) the use of NaBr (instead of NaCl) was introduced for isolation of nucleic acids. The bromide salt was of great advantage because it is very soluble in ethanol and hence does not coprecipitate with fibrous DNA when ethanol is added. The use of NaBr has a further advantage: in the case of nervous tissue, when M-NaCl was used for DNA isolation, enzymic depolymerization reduced the DNA yields to very small amounts, even in the presence of arsenate ion. But when a highly concentrated NaBr (~7 M) solution was used, such enzymic activity vanished, because the nucleases were salted out. It was the use of saturated solutions of NaBr that enabled us to obtain excellent yields of a highly polymerized product.

SUMMARY

1. A convenient method for isolation of fibrous DNA from cattle cerebellum is described.

2. A comparison was made of the chemical, spectrophotometric, viscometric, and polarimetric properties of DNA samples isolated from the cerebellum, kidney, and spleen.

3. Fibrous DNA isolated from the cerebellum resembled DNA prepared from kidney and spleen in chemical constitution and in the values obtained for the following physical measurements made in dilute and concentrated salt solutions: ultraviolet hyperchromicity and specific rotation.

4. The viscosity of cerebellum DNA was nearly twice that of DNA obtained from kidney and spleen.

5. The significance of these findings is discussed. It is suggested that cell constancy of the central nervous system does not depend on chemical constitution of its DNA.

6. A new phenomenon, high-salt hyperchromicity, was observed and is briefly discussed.

REFERENCES

BENDICH A., PAHL H. B. and BEISER S. M. (1956) *Cold Spr. Harb. Symp. quant. Biol.* **21,** 31.

BENDICH A., PAHL H. B., KORNGOLD G. C., ROSENKRANZ H. S. and FRESCO J. R. (1958) *J. Amer. chem. Soc.* **80,** 3949.

BLOCH D. P. (1958) In *Frontiers in Cytology* (Edited by PALAY S. L.) pp. 113–116. Yale University Press.

BLOUT E. R. and ASADOURIAN A. (1954) *Biochim. biophys. acta* **13,** 161.

DISCHE Z. (1955) In *The Nucleic Acids* (Edited by CHARGAFF E. and DAVIDSON J. N.) Vol. 1, p. 285. Academic Press, New York.

EMANUEL C. F. and CHAIKOFF I. L. (1957*a*) *Biochim. biophys. acta* **24,** 261.

EMANUEL C. F. and CHAIKOFF I. L. (1957*b*) *Biochim. biophys. acta* **24,** 254.

EMANUEL C. F. and CHAIKOFF I. L. (1958*a*) *Biochim. biophys. acta* **28,** 550.

EMANUEL C. F. and CHAIKOFF I. L. (1958*b*) Unpublished Studies.

FEIGL F. (1954) *Spot Tests* (4th Ed.) Vol. 2, p. 209. Elsevier, Amsterdam.

LAWLEY P. D. (1956) *Biochim. biophys. acta* **21,** 481; **22,** 451; **19,** 160.

SHACK J. (1958) *J. biol. Chem.* **233,** 677.

STUMPF P. K. (1947) *J. biol. Chem.* **169,** 367.

TAMM C., HODES M. E. and CHARGAFF E. (1952) *J. biol. Chem.* **195,** 49.

THOMAS R. (1954) *Biochim. biophys. acta* **14,** 231.

WYATT G. R. (1951) *Biochem. J.* **48,** 584.

ZAMENHOF S. (1956) *Progress in Biophysics and Biophysical Chemistry* **6,** 85. Pergamon Press, London.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 245 to 252. Pergamon Press Ltd. Printed in Northern Ireland

FACTOR S—A SUBSTANCE WHICH EXCITES CRUSTACEAN MUSCLE

WILLIAM G. VAN DER KLOOT
Department of Pharmacology, New York University College of Medicine, New York, New York

(*Received* 28 *September* 1959)

THOUGH it has been assumed that the Crustacea must contain a molecule which acts as a neuro-muscular transmitter, until recently there seemed to be little chance of easily isolating the excitatory substance itself. The difficulty stems from the dual innervation of Crustacean muscles, which receive inhibitory as well as excitatory neurons. Consequently, any crude extract would be apt to contain both excitatory and inhibitory factors—complicating separations depending upon biological tests of the extracts.

This difficulty can be overcome by adding picrotoxin to the crude extracts because picrotoxin selectively blocks the inhibitory junctions (VAN DER KLOOT, ROBBINS and COOKE, 1958; ROBBINS and VAN DER KLOOT, 1958; GRUNDFEST, REUBEN and RICKLES, 1958). By using picrotoxin in this way, an excitatory substance has been isolated from extracts of Crustacea. The substance has been partially purified; since its structure is still unknown, I propose to name the stimulating substance Factor S (stimulatory).

It is highly improbable that acetylcholine serves as the neuromuscular transmitter in the Crustacea because acetylcholine, curare-like compounds, and anti-cholinesterases in pharmacological concentrations do not affect the muscle (ELLIS, THIENES and WIERSMA, 1942). It might be said that the neuromuscular junctions are shielded from applied drugs, but the prompt actions of γ-aminobutyric acid, picrotoxin, and Factor S argue against this interpretation.

So far only two substances are known to excite Crustacean muscle in anything approaching physiological concentrations. The first is L-glutamic acid, which excites in concentrations slightly below 10^{-4} M (ROBBINS, 1958; VAN HARREVELD, 1959). The second is carnitine, which is reported to excite in unspecified concentrations (GRUNDFEST, REUBEN and RICKLES, 1959). There is no evidence that either L-glutamic acid or carnitine is actually the neuromuscular transmitter.

EXPERIMENTAL

Animals. Crayfish, *Cambarus clarki*, were purchased and stored in running tap water before use. Lobsters, *Homarus americanus*, were used immediately after purchase.

Preparation of crude extracts. The animals or tissues were ground in a Waring blendor in either 10% trichloroacetic acid (TCA) or in 0·4 M-perchloric acid. The extract was filtered, and the precipitate washed with 5% TCA or 0·2 M-perchloric acid. TCA was removed by ether extraction; perchlorate was precipitated by neutralizing the extract with KOH.

Assay. Extracts were tested for activity on the closer muscle of the isolated crayfish claw. The opener muscle was transected and the extract was perfused in through the cut tip of the claw. During the assay the nerve to the muscle was stimulated through platinum electrodes about once every 10 sec with a train of impulses lasting for 100 msec. The frequency of stimulation was varied to suit the

preparation; usually it was between 30 and 60 per sec. The stimulation was included to make certain that the crude extracts did not block muscular contraction. The response of the muscle was measured isometrically by a strain gauge. The signal from the strain gauge was amplified and recorded by a Grass ink-writer.

Chromatography. All chromatograms were descending and were run in a room at 25 ± 2°. Whatman No. 1 paper was used unless otherwise noted.

Collection of perfusates. An isolated claw was clamped in a special holder. Platinum stimulating electrodes were inserted into the meropodite. The claw was perfused with van Harreveld's solution flowing in through the cut tip at a rate of about 1 ml/sec. The perfusate was collected as it dripped from the proximal end of the claw into 20% TCA. Stimulation during perfusion was at a frequency of 300 per sec.

Van Harreveld's solution. Made by adding 55 ml of 0·2 M-Tris-maleate buffer at pH 7·4–945 ml of a solution which contained: 12·0 g NaCl, 0·4 g KCl, 0·5 g $MgCl_2 \cdot 6H_2O$ and 1·5 g $CaCl_2$ (VAN HARREVELD, 1936; ELLIOTT and FLOREY, 1956).

RESULTS

Preliminary separations. Crude extracts of crayfish or lobster were evaporated to dryness *in vacuo* at 40°. The residue was dissolved in a small volume of 100% ethanol. The solution was evaporated to dryness and the residue was dissolved in van Harreveld's solution containing 10^{-3} M-picrotoxin. These extracts when perfused into the crayfish claw elicited a brief contraction of the closer muscle.

Obviously many compounds are included in the active, ethanol-soluble fraction. For this reason, other methods of purification were sought. An excitatory fraction could be prepared by applying the aluminium hydroxide absorption method devised by VON EULER (1948) for the isolation of catecholamines. It appears that at least 70 per cent of the excitatory activity of crude extracts is precipitated with the aluminum hydroxide. An extract of crayfish nerve cord was highly active in a dilution of 88 μg solid/ml van Harreveld's solution.

A method for continuously extracting amines from an alkaline solution through ether into 4% phosphoric acid (BLOCK, DURRUM, and ZWEIG, 1958) was also useful in partially purifying the crude extracts. After extraction for 24 hr the phosphoric acid solution was neutralized to pH 7 and then saturated with a mixture of K_3PO_4: Na_2SO_4 (1 : 6 w/w) and shaken with *n*-butanol. The butanol layer was separated, dried with Na_2SO_4, and a small quantity of gaseous HCl was passed through. The precipitate which formed was inactive and was discarded. The butanol was evaporated *in vacuo* at 40°. The residue was dissolved in van Harreveld's solution and was active at a dilution of 120 μg/ml.

Chromatography. The active extracts prepared by aluminium hydroxide precipitation and by amine extraction were chromatographed in a variety of solvent systems. The spots were detected by exposing the developed chromatograms to cyanogen bromide vapours and spraying with aminoacetophenone, or by viewing the chromatograms in UV light after exposure to the vapours of ammonia and 2-butanone (the reason for selecting these techniques is given below). In some instances the developed chromatograms were cut into sections, the sections eluted, and the eluates tested for excitatory activity. The results of some of the experiments are summarized in Table 1. A number of R_F values found in each solvent system are included to show variations encountered. There is an excellent agreement between the location of the spot and the excitatory activity on the chromatogram. There is little doubt that the substance revealed by spraying the chromatogram is the excitatory

VOL. 5 1959/6

TABLE 1.—CHROMATOGRAPHY OF ACTIVE EXTRACTS

Solvent system	R_F of spot	Excitatory activity found between R_F's
Acetone: water (60 : 40)	0·64 0·68 0·67 0·70	Not tested
n-Butanol : acetic acid: acetone : water (5 : 1 : 1 : 3)	0·60 0·54	0·44–0·55
n-Butanol: acetic acid: water (4 : 1 : 5)	0·65	0·61–0·80
Phenol: 0·1 N-HCl (8 : 2)	0·04 0·09 0·06	0·0–0·14
n-Butyric acid: water: NaOH (69 : 31 : 0·85 w/v)	0·61 0·64 0·65	0·54–0·82
Pyridine: water (2 : 1)	0·74 0·71 0·72 0·70 0·74	Not tested
n-Propyl alcohol: 10% formic acid (80 : 40)	0·66 0·63 0·63	0·51–0·68
1-Butyric acid: water: NH_4OH (661 : 329 : 10) pH = 3·7	0·52 0·62 0·54 0·53 0·58	Not tested

factor and, therefore, it is called Factor S. A search of the literature has not disclosed a compound with chromatographic properties identical to those of Factor S.

Chemical reactions of Factor S. To study the chemical nature of Factor S the spots on the chromatograms were tested with 33 different spray and dip tests. After it was found that Factor S is absorbed on aluminium hydroxide, the chromatograms were tested by spraying with alkaline potassium ferricyanide. The Factor S spots colour pink with this reagent and yellow with napthaquinone 4-sulphonate; both reactions are typical of catecholamines (JAMES, 1948; GLAZKO and DILL, 1951). Nevertheless, Factor S does not appear to be a catecholamine, because it does not react with the Folin or with the Folin-Dennis spray reagents described by DALGLIESCH (1952).

Factor S produced a yellow spot when the chromatograms were tested with the method of CUTHBERTSON, IRELAND and WOLFF (1953) for revealing pyridine and piperidine compounds. The chromatograms were exposed to CNBr vapours followed by spraying with 1% aminoacetophenone in ethanol: water (1 : 1) or by dipping in aminoacetophenone in acetone. In following this lead, it was important to know whether or not Factor S is a pyridinium compound. This question was answered by viewing chromatograms in UV light after exposure to cyanide fumes or to the vapours of NH_4OH and 2-butanone. Factor S was revealed by both of these methods. The reactions with cyanide and with 2-butanone are typical of pyridinium compounds (BURTON, 1954; KODICEK and REDDI, 1951).

Factor S spots also reacted with two reagents for phosphate; the molybdate method of HANES and ISHERWOOD (1949) and the ferric chloride-salicylsulphonic acid method of WADE and MORGAN (1953). However, in my hands nicotinamide is also coloured by these methods, so it cannot be decided whether Factor S contains phosphate.

Purification by column chromatography. Further purification of Factor S was obtained by absorbing partially purified extracts on Amberlite IRC 50 cation exchange resin in the sodium form. The columns were washed with 0·05 N-HCl. With a column 10 × 220 mm, for example, Factor S began to appear in the eluate after 400 ml of HCl had passed through the column, and elution was substantially completed with the next 50 ml of solvent. The tubes containing the eluates were dried *in vacuo*, the residue was taken up in crayfish Ringer solution and tested on the closer muscle. The material in the tube containing the eluate from 430–440 ml was the most active: when this fraction was tested on the muscle it was excitatory when diluted to 12 μg/ml.

Purification by electrochromatography. Factor S was also purified by continuous electrochromatography in the apparatus manufactured by Microchemical Specialties Corp. The solvent was 0·25 N-acetic acid; the current flow was 1 mA. The course of the compound across the curtain was charted by drying the paper and exposing the chromatogram to ammonia–2-butanone. As judged from the developed curtain, a substance which reacted with ammonia–2-butanone migrated about 20 mm toward the anode and was collected almost entirely in a single tube. The solvent was evaporated from all the tubes and the residues were tested; only the tube to which the reactive substance had been traced contained material having excitatory activity (Fig. 1).

Absorption spectra. The Factor S, which had been isolated by ion-exchange chromatography and by electro-chromatography, was studied by measuring the absorption spectrum in a Beckman spectrophotometer. The only significant absorption was in the UV (Fig. 2). The principal peak is around 260 mμ. There was little change in the absorption when the extracts were treated with sodium bisulphite, or with ethanol and yeast ethanol dehydrogenase—DPN and TPN are reduced by these reagents and yield a new peak at 340 mμ. On the other hand, when 1 ml of either of the Factor S extracts was mixed in the cuvette with 2 ml of N-KCN, a sharp peak appeared at 327 mμ (Fig. 2). The appearance of a peak at this wavelength following treatment with cyanide is characteristic of 1-substituted nicotinamide derivatives (COLOWICK, KAPLAN and CIOTTI, 1951).

The incorporation of labelled nicotinic acid. The evidence so far suggests that Factor S is a derivative of nicotinic acid. Therefore, a crayfish was injected with

VOL. 5 1959/6

0·5 μC of nicotinic acid-7-^{14}C. One day later, the animal was sacrificed and extracted by the aluminium hydroxide method. The extract was spotted on a strip of Whatman No. 3 filter paper and the chromatogram was developed in isobutyric acid: water: NH_4OH (see Table 1). The dried strip was counted on a recording chromatogram

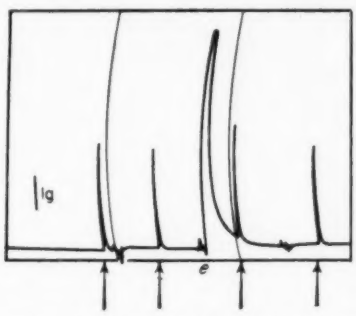


FIG. 1.—The test of an active extract prepared by electrochromatography. The abscissa represents time; each scale division is 5 sec. The ordinate records the tension of the closer muscle of the crayfish claw. At the arrows the motor nerve was stimulated at a frequency of 30 per sec for 100 msec. At 'e' the active extract was perfused into the claw.

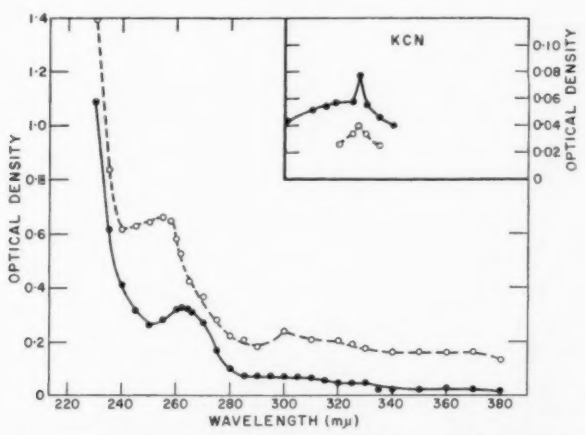


FIG. 2.—The absorption spectra of two extracts containing Factor S. ●——● extract prepared by ion-exchange chromatography. ○——○ extract prepared by electrochromatography (the optical density values for this extract are multiplied by ten). The insert shows the absorption from 300 to 340 mμ in 0·66 M-KCN.

scanner (Fig. 3). The radio-activity was localized in a peak at R_F 0·53, and thus had the same mobility as Factor S in this solvent system.

Factor S in perfusates from stimulated claws. Factor S has been studied so extensively because it seems possible that it is a neuromuscular transmitter in the Crustacea. If this is true, then this substance should be detected in perfusates obtained from claws during stimulation of the motor nerves, To this end, perfusates were collected from stimulated claws (see Experimental), treated with ether to remove the TCA, neutralized with NaOH, saturated with K_3PO_4 : Na_2SO_4 (1 : 6 w/w), and shaken with *n*-butanol. The butanol layer was separated, dried, treated with gaseous HCl, and evaporated to dryness. The residue, when taken up in van Harreveld's

solution, was highly active in exciting the crayfish closer muscle. Chromatography showed that the perfusate contained a substance which coloured with CNBr–amino-acetophenone and with 2-butanone–ammonia. The material in the perfusate moved to the same position on chromatograms, when tested in four different solvent systems, as the Factor S prepared from extracts of whole animals. It can be concluded that the perfusate from stimulated muscles contains Factor S. So far this substance has not been detected in perfusates of unstimulated muscles.

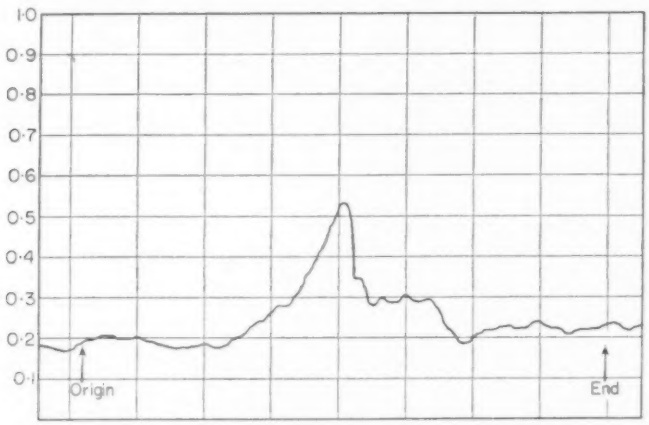


FIG. 3.—The distribution of radioactivity on a chromatogram of an aluminium hydroxide extract of a crayfish injected 24 hr before sacrifice with nicotinic acid-7-^{14}C. Full scale is 300 counts per min. The time constant of the scanner was 25 sec.

DISCUSSION

Perhaps the most surprising finding is that when a variety of different methods was used for the partial purification of excitatory extracts from Crustacea, the same product always appeared at the end. Such diverse manoeuvres as aluminium hydroxide precipitation, amine extraction, ion-exchange chromatography, and electrochromatography always yielded a product which reacted with cyanogen bromide and which had the characteristic chromatographic properties summarized in Table 1. The excitatory material which has been isolated is called Factor S.

The evidence shows that Factor S is a derivative of pyridine or piperidine as judged by its reaction with cyanogen bromide. The reaction with 2-butanone and ammonia marks it as a 1-substituted derivative. The appearance of a peak in the absorption spectrum of Factor S which has been treated with cyanide supports the interpretation that it is a pyridinium salt. Moreover, COLOWICK, KAPLAN and CIOTTI (1951) found that while the cyanide reaction is seen with 1-methylnicotinamide, it is not found with 1-methylnicotinic acid (trigonelline). This finding was interpreted to mean that the amide group, in addition to a substitution on the ring nitrogen, is required for the cyanide reaction. The sum of the evidence, therefore, suggests that Factor S is a 1-substituted derivative of nicotinamide. The group substituted on the nitrogen of the pyridine ring remains unknown. The next step in the investigation will be the hydrolysis of Factor S and an attempt to characterize the liberated parts of the molecule.

During this study, a considerable number of compounds was tested on the crayfish muscle in the hope that they might sufficiently resemble Factor S to possess physiological activity. A few of the more obvious ones which were ineffective are recorded (Table 2). So far in this study, the only known compounds found to be active are 1-alkyl derivatives of nikethamide (N : N-diethylnicotinamide), which block neuromuscular transmission. The fashion in which these compounds act is now being studied.

TABLE 2.—SOME SUBSTANCES WHICH DO NOT EXCITE OR BLOCK THE CONTRACTION OF THE CLOSER MUSCLE OF THE CRAYFISH CLAW AT 10^{-2}M

Diphosphopyridine nucleotide (DPN)	Nicotinic acid
TPN	Nicotinamide
DPNH	Nikethamide
TPNH	Pyridoxamine
Nicotinamide mononucleotide	Pyridoxal phosphate
Trigonelline	Pyridoxal
1-methyl nicotinamide chloride	

From the literature, I have found only one compound which might be identical to Factor S, namely, the 'Catechol-4' discovered by ÖSTLUND (1954). This substance, like Factor S, is extracted by aluminium hydroxide precipitation, coloured with ferricyanide, and has a low R_F value in phenol: 0·1 N-HCl. 'Catechol-4' was found in several insects, in the colenterate, *Metridium dianthis*, the protozoan, *Noctiluca miliaris*, and in the mollusc, *Mytilus edulis*. ÖSTLUND did not find 'Catechol-4' in extracts of the shrimp, *Cragon cragon*. So far I have found Factor S in crayfish, lobsters, the pupa of *Hyalophora cecropia*, and in the brains of the rat and the cow. Further studies of its distribution, now underway, should clarify the relation, if any, to the 'Catechol-4' of ÖSTLUND.

A point from this study which is of interest to general neurochemistry is that Factor S is precipitated by the aluminium hydroxide procedure devised for catecholamines. Until now, the aluminium hydroxide procedure has been regarded as relatively specific for catecholamines, but there is no reason to believe that Factor S is a catechol.

The evidence here presented does not prove that Factor S is a neuromuscular transmitter in the Crustacea. Nevertheless, the evidence supporting this interpretation is already compelling. Factor S excites the muscle in low concentrations. Moreover, the contraction produced when it is perfused into the claw is transient—the muscle recovers rapidly. The transitory action of Factor S may be owing in part to diffusion, but the results also hint that the molecule may be rapidly destroyed by the tissues. It is apparent that Crustaceans are able to synthesize this compound because they contain the molecule and because they are able to incorporate labelled nicotinic acid into Factor S. A further argument for believing that Factor S may serve as a transmitter is the presence of this compound in perfusates of stimulated muscles; apparently during stimulation it is released by either the muscle or by the nerve.

SUMMARY

A substance was found in extracts of crayfish and of lobsters which in low concentration stimulates the contraction of the closer muscle of the claw. This substance

is called Factor S. Factor S has been partially purified by aluminium hydroxide precipitation, amine extraction, and by paper, ion-exchange, and electro-chromatography. The reactions of the substance on paper chromatograms and the absorption spectra suggest that it is a 1-substituted derivative of nicotinamide. Factor S was detected in perfusates from stimulated claws, but was not detected in perfusates from unstimulated claws. It is suggested that Factor S is a neuromuscular transmitter in the Crustacea and that it may also be important in the physiology of other animals.

Acknowledgement—This investigation was supported by research grant, B-1870, from the Institute of Neurological Diseases and Blindness, Public Health Service. I am grateful to HAROLD GORDON for technical assistance.

REFERENCES

BLOCK R. J., DURRUM E. L. and ZWEIG W. (1958) *Paper Chromatography and Paper Electrophoresis.* Academic Press, New York.
BURTON H. S. (1954) *Nature, Lond.* **173,** 127.
COLOWICK S. P., KAPLAN N. O. and CIOTTI M. M. (1951) *J. biol. Chem.* **191,** 447.
CUTHBERTSON W. F. J., IRELAND D. M. and WOLFF W. (1953) *Biochem. J.* **55,** 669.
DALGLIESH C. E. (1952) *J. Chem. Soc.* 3940.
ELLIOTT K. A. C. and FLOREY E. (1956) *J. Neurochem.* **1,** 181.
ELLIS C. H., THIENES C. H. and WIERSMA C. A. G. (1942) *Biol. Bull. Woods Hole* **83,** 334.
EULER U. S. VON (1948) *Arch. int. Pharmacodyn.* **77,** 477.
GLAZKO A. J. and DILL W. A. (1951) *Nature, Lond.* **168,** 32.
GRUNDFEST H., REUBEN J. P. and RICKLES W. H., JR. (1958) *Biol. Bull., Woods Hole* **115,** 332.
GRUNDFEST H., REUBEN J. P. and RICKLES W. H. JR. (1959), *J. gen. Physiol.* **42,** 1301.
HANES C. S. and ISHERWOOD F. A. (1949) *Nature, Lond.* **164,** 1107.
JAMES W. O. (1948) *Nature, Lond.* **161,** 851.
KODICEK E. and REDDI K. K. (1951) *Nature, Lond.* **168,** 475.
ÖSTLUND E. (1954) *Acta physiol. scand.* **31,** Suppl. 112, 1.
ROBBINS J. (1958) *Anat. Rec.* **132,** 492.
ROBBINS J. and VAN DER KLOOT W. G. (1958) *J. Physiol.* **143,** 541.
WADE H. E. and MORGAN D. M. (1953) *Nature, Lond.* **171,** 529.
VAN DER KLOOT W. G., ROBBINS J. and COOKE I. (1958) *Science* **127,** 521.
VAN HARREVELD A. (1936) *Proc. Soc. exp. Biol., N. Y.* **34,** 428.
VAN HARREVELD A. (1959) in *Inhibition in the C. N. S. and Gamma-aminobutyric Acid.* Pergamon Press, London (in the press).

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 253 to 276. Pergamon Press Ltd. Printed in Northern Ireland

GLUCOSE CATABOLISM OF RABBIT RETINA BEFORE AND AFTER DEVELOPMENT OF VISUAL FUNCTION*

LEONARD H. COHEN† and WERNER K. NOELL
Roswell Park Memorial Institute, Buffalo, New York

(*Received* 8 *September* 1959)

OL.
5
9/60

THE significance of glucose in the metabolism of the mammalian retina is attested by the high rate of aerobic glycolysis of this tissue *in vitro* (WARBURG, POSENER and NEGELEIN, 1924) and is further indicated by the extraordinary susceptibility of the visual cells to iodoacetate *in vivo* (NOELL, 1951, 1952). The retina is also known to respire rapidly (WARBURG, 1927) but it is undetermined to what extent this respiration is supported by glucose oxidation. As an approach to some of the questions raised by these observations we have studied the metabolic fate of glucose in the isolated retina. To provide a possible insight into the relation of glucose metabolism to retinal function, the fully developed adult rabbit retina was compared with the young tissue at 5 to 7 days after birth, a time when the retina is not yet functioning and when the rods and cones are still small, growing organelles (NOELL, 1958*a*, 1958*b*). The rabbit retina was chosen because this animal has been especially useful in electrophysiological studies of the retina and because much work has been done on the effects of metabolic inhibitors upon its *in vivo* functions (NOELL, 1953, 1958). The fine structure of the rabbit's visual cells has been studied extensively by electron microscopy (DEROBERTIS and FRANCHI, 1956; DEROBERTIS, 1956; DEROBERTIS and LASANSKY, 1958) including the changes in ultrastructure resulting from iodoacetate (LASANSKY and DEROBERTIS, 1959). The distribution of key enzymes of glucose metabolism over the various layers of the rabbit's retina has been determined by LOWRY, ROBERTS and LEWIS (1956).

EXPERIMENTAL AND METHODS

Experimental procedure

The retinas used were all from New Zealand White rabbits. 'Adult' retinas were from animals 6–9 months old, and 'young' ones from those not older than 1 week. The eye was quickly removed after decapitation and transferred to a special cup of perforated stainless steel where it could be held in place by gentle suction. The front portion of the eye was sliced off just posterior to the ora serrata. The posterior hemisphere was everted on a suitably-shaped rubber stopper, pinned down at the periphery, and immersed in substrate-free Krebs–Ringer-phosphate. The retina was gently teased free with a smooth glass spatula and then dropped into a prepared Warburg flask by severing the optic nerve. The time required for the entire procedure was 3–5 min. Each Warburg flask contained one whole retina; it was immediately affixed to a manometer and equilibrated at 37° for 10 min. In

* This study was supported by the U.S. Public Health Grant B 812. Preliminary reports of this work were presented at meetings of the American Society of Biological Chemists and The American Physiological Society (COHEN, 1957; COHEN and NOELL, 1958; COHEN and NOELL, 1959), and at the Symposia on Fundamental Aspects of Vision (NOELL, 1958*a*) and Photoreception (NOELL, 1958*b*).

† Present address: Dept. of Pharmacology, University of Manitoba, Winnipeg, Canada.

some experiments the Warburg flask and medium were kept warm until the retina was added, thereby permitting a reduction of the equilibration time to 5 min. Readings were taken for an hour, at which time the reaction was stopped by the addition of either trichloroacetic acid or perchloric acid from the side arm. When ^{14}C-labelled glucose was used as substrate shaking was continued for 20 min after the addition of acid to insure complete absorption of radioactive CO_2 by NaOH in the centre well. A portion of the medium was neutralized with NaOH and assayed for lactate. The remainder of the flask contents was filtered through a small, weighed disc of filter paper (Whatman 41H), dried over $CaCl_2$ *in vacuo*, weighed, and a correction applied for the salts retained by the filter paper. Conventional Warburg flasks with a reaction volume of 3 ml were used for adult retinas (dry weight about 10 mg). Control experiments for lactic acid production were performed in 30 ml of medium. For young retinas (dry weight about 5 mg) smaller vessels were used (approximately 7 ml) and the volume of the medium was 1·5 ml.

The incubation medium had the following composition: 149 mM-Na^+; 6·46 mM-K^+; 1·24 mM-Ca^{2+}; 1·3 mM-Mg^{2+}; 139 mM-Cl^-; 1·3 mM-SO_4^{2-}; 11·4 mM-$[H_2PO_4^- + HPO_4^{2-}]$; pH 7·4. This is essentially Krebs–Ringer-phosphate buffer except that the Ca^{2+} concentration is that of cerebrospinal fluid (MERRITT and FREMONT-SMITH, 1938). By using the lower Ca^{2+} concentration, the occasional formation of a precipitate was eliminated without affecting the results. In experiments where the K^+ concentration of the medium was altered, the change was compensated for by a change in Na^+ concentration. Routinely, additions to the medium were made prior to the incubation of the retina. Glucose was added to a final concentration of 20 mM; sodium pyruvate to a final concentration of 10 mM. The gas phase was air or purified nitrogen (Linde, specified 99·99% N_2) passed through three successive solutions of alkaline anthraquinone-hydrosulphite (FIESER, 1950) containing 2 drops of *antifoam* 60 (General Electric Co.) and through water. Air was preferred to 100% O_2 because of the damaging effect of high O_2 concentrations on the rabbit's retina *in vivo* (NOELL, 1958). Control experiments on adult retinas were performed with 60% O_2; in comparison to the measurements in air no differences were found for the rates of respiration, glucose oxidation, and glycolysis, with the exception of a 10–20 per cent greater stimulation of respiration and glucose oxidation by 2 : 4-dinitrophenol.

The CO_2 production by the adult retina in the phosphate-buffered medium was determined by differential manometry as described by SUMMERSON (1939). CO_2 production by the young retina was measured by comparing the respiration with and without NaOH in the centre well (DIXON, 1951) and applying a correction for the $NaHCO_3$ present at the end of the equilibration period.

The total amount of CO_2 originating from the glucose of the medium was determined from measurements of the radioactivity of the CO_2 formed when retinas were incubated with uniformly labelled glucose (glucose-U-C^{14}). The amount of CO_2 arising from the carbon at a particular position was determined by using glucose labelled in that position only.

The specific activities of the labelled glucose used were adjusted, on the basis of preliminary experiments, so that the CO_2 formed yielded counting rates of at least 200 counts/min above background except in cases of extremely low metabolic activity.

In experiments designed to equilibrate the endogenous stores of carbohydrates and intermediary metabolites with the labelled glucose before the measurement of glucose oxidation was begun, the excised retinas were preincubated for various periods of time in a medium containing radioactive glucose and then transferred to Warburg flasks containing radioactive glucose of the same specific activity.

Analytical

Lactate determination. Lactate was measured spectrophotometrically with lactic dehydrogenase and DPN, by the method of HORN and BRUN (1956) adapted for routine microassays. Samples of 30–200 μl of the neutralized solutions to be assayed were pipetted into 10 × 75 mm test tubes. A standard series was prepared containing 0–30 μl of 6 mM-L-lactate (3 mM-zinc lactate*) per tube. The volumes in all tubes were adjusted with water to that of the largest sample, and to each was added 600 μl of a solution of DPN–semicarbazide-buffer (freshly prepared by mixing 1 part of a solution containing 15 mg DPN·$4H_2O$/ml with 5 parts of a 6% solution of semicarbazide in 0·25 M-glycine

* Zinc lactate, Fisher catalogue No. 2–43, although listed as 'inactive', was found to react quantitatively as L-lactate in the lactic dehydrogenase reaction. Correspondence with the Fisher Scientific Company confirmed the fact that it was the L-isomer.

VOL. 5 1959/6

buffer, pH 10·6) and 25 μl of a solution of lactic dehydrogenase. Commercial lactic dehydrogenase (twice recrystallized) was obtained as a suspension in $(NH_4)_2\,SO_4$ solution and was diluted before use with an equal volume of 6% $NaHCO_3$. The tubes were sealed with parafilm, shaken, and allowed to stand at room temperature for at least 2 hr. The absorbancy of each sample at 366 mμ was read in a microcuvette against the blank sample of the standard curve, with a Beckman spectrophotometer, model DU. Since the blank was found to be pH-dependent, each test tube was kept covered until its contents were read, and the blank cuvette was sealed with parafilm in order to exclude CO_2. The standard curve was usually linear and coincided with that calculated from the molar absorbancy of DPNH. The method was found to be precise within $\pm$ 2% and to agree within $\pm$ 2·5% with the manometric determination of lactic acid production. The assay was unaffected by the presence, in the samples assayed, of 2×10^{-3} M-iodoacetate or 10^{-2} M-pyruvate. Dinitrophenol contributed sufficient absorbancy to necessitate a small correction, but did not otherwise affect the assay.

Pyruvate analysis. Pyruvate was assayed spectrophotometrically by its reaction with DPNH catalysed by lactic dehydrogenase. Neutralized samples or the unknown solution of the standard series (200 μl total volume) containing 15–50 mμmoles pyruvate were mixed in the cuvette with 100 μl of DPNH solution (0·6 mg disodium DPNH/ml in 0·1 M-sodium phosphate, pH 6·5) and 5 μl of lactic dehydrogenase (diluted 1 : 1 with 3% $NaHCO_3$). After 5 min their optical densities were measured at 340 mμ against the standard containing the largest amount of pyruvate. The pyruvate content was read by comparison with the standard curve.

Measurement of radioactivity (i) In order to count the metabolic CO_2, the contents of the centre well of the Warburg flask were added quantitively to 0·6 m-moles Na_2CO_3 and precipitated with an excess of $BaCl_2$, and the $BaCO_3$ was counted at infinite thinness for 2560 counts in a gas flow counter with micromil window.

(ii) To obtain the 'specific activity' of the labelled carbons of glucose used as substrate, a sample (0·5–5 μmoles) was combusted to CO_2 by the persulphate oxidation method (CALVIN *et al.*, 1949), adapted for use with Warburg flasks. The Na_2CO_3 so obtained was mixed with 0·6 m-moles of Na_2CO_3 and counted exactly as described above for metabolic CO_2, but for 10,000 counts.

(iii) Metabolic lactate was isolated from the incubation mixture as described below and was combusted and counted in the same manner as were the glucose standards.

(iv) Glutamic acid isolated from the incubation mixture was counted at infinite thinness.

Isolation of lactate and glutamate by anion exchange chromatography. At the end of the manometric experiment the flasks were kept in the cold overnight. The retina was removed and washed several times with 1 ml portions of 1% trichloroacetic acid. The combined washings and flask contents were made pink to phenolphthalein, diluted with 40 ml of water, and run through a column of Dowex-1-chloride $\times$ 4, 1 cm diameter $\times$ 7 cm length. The glucose was washed through with 30 ml of water and elution was begun with a linear HCl gradient from 0–0·02 N over a volume of 400 ml (LINDBERG and WICKBERG, 1954). Fractions of 3–4 ml were collected. The first peak of radioactivity, which appeared after 40–45 ml of eluant had passed through the column was shown by paper chromatography to contain glutamate, traces of aspartate, and other radioactive materials. The second peak, containing lactate, followed the first by 5–10 ml. The lactate was eluted in 4 or 5 fractions. The specific activity (counts/min per mμmole lactate carbon) of each fraction was measured separately and only the specific activity of the fraction containing the most lactate was used. Actually, all of the lactate fractions of any one chromatogram had the same specific activity except the 'tails', in which the specific activity was 10% higher.

After elution of the lactic acid, pyruvate was eluted with a few ml of solution of 0·01 N–HCl and M–NaCl. Of pyruvate added to the column, 97% was recovered in this fraction.

The fraction containing glutamate was evaporated to dryness and taken up in 1 ml water. Of this solution, 0·5 ml was added to 50 mg carrier glutamic acid which was dissolved by neutralization and recrystallized by acidification. The recrystallization was repeated two more times. The crystalline material was plated at several thicknesses, counted as described above, and the activity extrapolated to infinite thinness. In some experiments, the glutamate fraction was chromatographed on paper with 80% phenol prior to the addition of unlabelled glutamic acid but without significantly different results.

Materials. Lactic dehydrogenase (twice recrystallized) and DPN were obtained from the Sigma Chemical Co. Glucose-U-^{14}C, glucose-1-^{14}C and glucose-6-^{14}C were obtained from New England Nuclear Corp. and glucose-2-^{14}C from the National Bureau of Standards.

RESULTS

I. *Rates of Glycolysis, Respiration, and Glucose Oxidation*

Young retina

With 0·02 M-glucose as substrate, aerobic lactic acid formation of the young retina was very high, amounting to almost 500 mμmoles/mg dry weight per hour (Table 1). There was nevertheless a marked Pasteur effect, anaerobic lactic acid production being 2·2 times greater than the aerobic rate. Respiration was quite low. Expressed in conventional Q notations, (μl/mg dry wt per hr) the mean values were $Q_{LA}^{Air} = 10{\cdot}8$; $Q_{LA}^{N_2} = 24$; $Q_{O_2} = -3{\cdot}4$. The initial rates for lactic acid production, determined by removing samples after various periods of incubation, were approximately twice as high as these.

TABLE 1.—YOUNG RETINA: GLYCOLYSIS, RESPIRATION AND GLUCOSE OXIDATION
Results are expressed as means ± S.E. The numbers of measurements are indicated in parenthesis. Incubation was for 70 min.

Conditions	Anaerobic	Aerobic			
	Lactic acid	Lactic acid	Oxygen uptake	CO_2 from glucose-U-^{14}C	
	(mμmoles/mg dry wt per hr)				(% of respiration)
20 mM-Glucose	1080 ± 44(5)	482 ± 8(21)	152 ± 3(28)	44 ± 2(26)	29·6 ± 1·3(26)
No substrate	122 ± 6(3)	50 ± 9(5)	235 ± 6(12)	—	—
20 mM-Glucose 2×10^{-3} M-IAA	72 ± 5(2)	48 ± 5(12)	138 ± 8(12)	5 ± 1(2)	4 ± 1(2)
20 mM-Glucose No Ca^{2+}	—	445 ± 6(4)	170 ± 12(9)	54 ± 3(9)	30 ± 0·8(9)
20 mM-Glucose 5×10^{-5} M-DNP	—	636 ± 20(4)	323 ± 20(4)	134 ± 6(3)	41 ± 1·4(3)

With no substrate in the medium, the amount of lactic acid formed by the young retina was very small (Fig. 1) and was almost all produced during the first 15 min of incubation. Respiration without added substrate was nevertheless linear and, as known also for cancer tissue, was significantly higher than it was with glucose present. The average respiratory decrease produced by glucose ('Crabtree effect') amounted to 35 per cent (Table 1), as compared with values of 40–60 per cent reported for ascites tumours (KUN, TALALAY and WILLIAMS-ASHMAN, 1951; BRIN and MCKEE, 1956; RACKER, 1956). In agreement with the ascites cell studies (RACKER, 1956) the Crabtree effect was not apparent in the presence of 5×10^{-5} M-2:4-dinitrophenol.

Although glucose inhibited O_2 uptake, it was oxidized to CO_2. The mean rate at

which CO_2 was formed from glucose carbon was 44 mμmoles/mg dry wt per hr when the mean O_2 uptake was 150 mμmoles (Table 1). If it is assumed that one molecule of O_2 is consumed for every molecule of CO_2 formed from glucose, then only 30 per cent of the respiration of the young retina is accounted for by glucose oxidation. This low contribution of glucose oxidation to respiration was also found in the experiments in which the retina was preincubated for up to 40 min with ^{14}C glucose, a finding which argues against a preferential oxidation of endogenous glucose. The total production

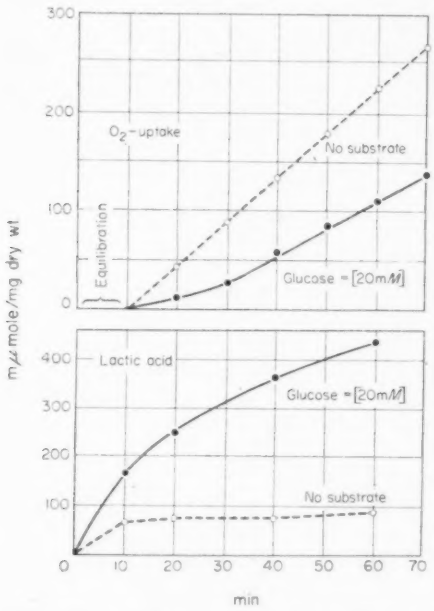


FIG. 1.—Young retina: Effects of glucose on respiration and lactic acid production. Medium: Krebs–Ringer-phosphate; gas phase: air. Four different flasks each containing one retina; reaction volume: 2·4 ml. Lactic acid production was measured by withdrawing small samples of medium for lactate determination at various intervals during incubation.

of CO_2, measured in two experiments, was equivalent to 88 per cent and 94 per cent of the O_2 consumed. Although these data are not sufficient to establish the R.Q. of the young retina, they indicate that not more than about 35 per cent of the total CO_2 produced comes from glucose carbon. In contrast, glucose was the sole source of lactate carbon, since the specific activity of the lactic acid formed was the same as that of the glucose of the medium (Table 4). These facts, considered together, strongly suggest that the large excess of oxygen consumption over $^{14}CO_2$ production was due to the oxidation of endogenous substrates other than glycogen, glucose, or glycolytic products.

This conclusion is supported by the finding that retinal respiration at this age is insensitive to iodoacetate (Table 1). While inhibiting glycolysis by 90 per cent, iodoacetate depressed O_2 uptake on the average by only 10 per cent. Glucose oxidation measured with ^{14}C glucose, however, was as much depressed as was glycolysis; the substrates for the resistant respiration, therefore, were probably not carbohydrates.

In contrast to nervous tissue generally and the fully developed retina in particular (see below), the omission of Ca^{2+} from the medium had little effect upon the young retina. However, 2:4-dinitrophenol (DNP) at 5×10^{-5} M increased respiration by 120 per cent and glucose oxidation by 200 per cent.

Adult retina

The dry weight of the fully developed retina is about twice that of the young but in cell number the young and adult are about the same since there is no cell division

TABLE 2.—ADULT RETINA: GLYCOLYSIS, RESPIRATION AND GLUCOSE OXIDATION
Results are expressed as means ± S.E. The numbers of measurements are indicated in parenthesis. Incubation was for 70 min.

Conditions	Anaerobic	Aerobic			
	Lactic acid	Lactic acid	Oxygen uptake	CO_2 from glucose-U-^{14}C	
	(mμmoles/mg dry wt per hr)				(% of respiration)
20 mM-Glucose	1340 ± 29(10)	856 ± 13(20)	448 ± 9(28)	307 ± 21(18)	68 ± 1·45(18)
No substrate	467 ± 35(4)	240 ± 31(5)	376 ± 18(5)	—	—
20 mM-Glucose 2×10^{-3} M-IAA	139 ± 5(4)	63 ± 4(4)	167 ± 7(4)	10·4 ± 0·29(2)	6 ± 0·7(2)
20 mM-Glucose No Ca^{2+}	—	981 ± 21(14)	863 ± 15(14)	718 ± 27(4)	81 ± 1(4)
20 mM-Glucose 5×10^{-5} M-DNP	1590 ± 30(5)	1340 ± 37(5)	1030 ± 17(5)	836 ± 21(5)	81 ± 1·5(5)
20 mM-Glucose High K^+ (27 mM)	−1%(4)	+4%(5)	−42%(5)	−50%(5)	53
20 mM-Glucose No K^+	−11%(2)	−11%(4)	−38%(4)	−42%(4)	61
20 mM-Glucose Light-adapted retina	—	−3%(2)	−2%(2)	−2%(2)	68

beyond two days after birth (NOELL, 1958*a*). On a dry weight basis all of the measured metabolic rates of the adult retina (Table 2) were higher than those of the young retina (Table 1) and these differences would appear larger if the measurement were related to cell number.

The capacity of the adult tissue to form lactic acid under anaerobic conditions

VOL. 5 1959/6

(Table 2) exceeded that of the young retina by approximately 25 per cent. Aerobically, the difference between young and adult was more marked, about 75 per cent, so that the Pasteur effect was smaller in the adult retina than in the young. Oxygen uptake by the adult tissue was 3-fold higher than that of the young retina and was about half the lactic acid production on a molar basis. In Q notation (μl/mg dry wt per hr) the mean values were $Q_{O_2} = -10$ and $Q_{LA}^{Air} = 19{\cdot}2$. Respiration was linear but lactate formation was not, and the rate measured over the initial 10 min of incubation corresponded to a Q_{LA}^{Air} ranging from 32 to 43. When the volume of the incubation

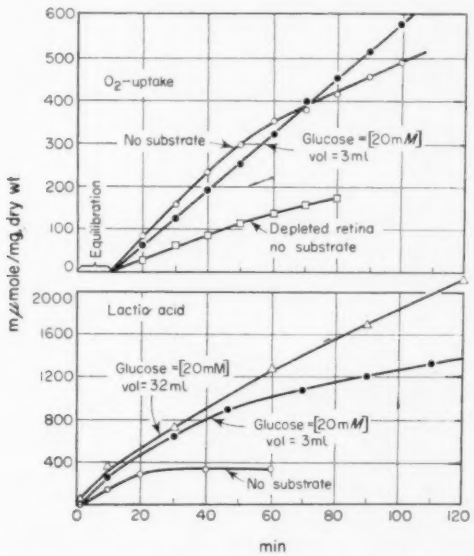


FIG. 2.—Adult retina: Effects of glucose on respiration and lactic acid production. Medium: Krebs–Ringer-phosphate; gas phase: air. Six different experiments. The comparison for O_2 uptake between glucose present and absent was performed on the two retinas of the same animal. The 'depleted retina' had been preincubated for 70 min in 10 ml glucose-free medium, then rinsed in fresh medium and transferred to the Warburg flask.

medium was increased to 10 times that usually used, aerobic lactic acid production was more linear and was about 30 per cent higher at the end of the usual 70 min of incubation (Fig. 2).

When glucose was omitted from the medium, the lactic acid produced anaerobically amounted to almost 500 mμmoles/mg dry wt, most of which was produced during the first 50 min of incubation. Hence, at the time the retina was transferred into the flask (see Experimental) its carbohydrate stores were at least the equivalent of 250 mμmoles hexose per mg dry wt. This exceeds by 6-fold the comparable stores in the young retina; it is also much greater than the sum of the glycogen hexoses and free hexoses of the brain which are reported to be less than 10 mμmoles per mg wet wt (MCILWAIN, 1955). Under aerobic conditions, lactic acid production from endogenous sources proceeded for 10–20 min at about half maximal rate and practically ceased after 40–60 min

The respiratory rate in a glucose-free medium was consistently a little higher

than that of the control retina (from the other eye of the animal) respiring in 0·02 M-glucose, but only during the first 20 min (Fig. 2). Thus a Crabtree effect of at most 10 per cent appeared to exist initially. Respiration soon decreased, however, and fell rather sharply at 30–50 min, at about the same time that lactate accumulation declined to a very low rate. Thereafter, respiration continued at a steady rate of about a third of that of the control eye. Upon the addition of ^{14}C glucose, respiration was stimulated to approximately the initial rate. This stimulation was entirely due to the oxidation of added glucose; in fact, more $^{14}CO_2$ was evolved than could be accounted for by the stimulation of O_2 uptake. The substrate of the slow prolonged phase of endogenous respiration was not the lactate produced initially because the retina continued to respire at the same rate for an additional 60 min when transferred to fresh medium after an initial 70 min incubation. These results suggest that the endogenous respiration includes two components, one of which is short-lived and probably supported by hexose stores, and the other one long-lived and maintained by non-carbohydrate substrates.

The oxidation of glucose of the medium to CO_2 was very rapid in the adult retina, exceeding that of the young retina by a factor of 6 (cf. Tables 1 and 2). Moreover, glucose oxidation was very high in relation to respiration. About 300 mμmoles of CO_2/mg dry wt per hr were produced from glucose, accounting for 68 per cent of the O_2 uptake, whereas the total CO_2 formed, as measured in four experiments, was 95–105 per cent of the O_2 consumed. Prolonged preincubation of the retina with labelled glucose (see Experimental) did not increase beyond 75 per cent the fraction of respiration accounted for by the oxidation of ^{14}C glucose.

As might be expected from the predominant utilization of glucose in respiration, iodoacetate had a marked inhibitory effect upon O_2 consumption. Doses of iodoacetate which blocked lactate production and glucose oxidation almost completely, inhibited respiration by about 60 per cent (Table 2).

Glucose oxidation of the adult retina was the most sensitive metabolic component to changes in the medium (Table 2). The omission of Ca^{2+}, which had little effect upon glycolysis, doubled respiration, and the increase was stoichiometrically accounted for by an increased formation of CO_2 from glucose. Dinitrophenol stimulated all measured activities and more than doubled respiration and glucose oxidation (see also Experimental). The effects of altering the K^+ concentration of the medium are expressed in Table 2 as percentage changes from the control flask values since they are the results of a separate series of experiments in which the average control values for aerobic glycolysis, respiration, and glucose oxidation were 5–10 per cent and anaerobic glycolysis 20 per cent lower than in the main series. Lactate formation was unaffected by the high K^+ concentration and slightly depressed by the omission of K^+ from the medium. Respiration, however, especially that due to glucose oxidation, was markedly depressed by either of these alterations in K^+ content. None of the metabolic parameters studied was affected by light adaption of the retina *in vivo* for 30 min to 10,000 lux, from which the electroretinogram recovers only after several hours (ELENIUS, 1958).

Development of glucose metabolism

It is apparent from the preceding section that the most striking difference between the two age groups is the high capacity of the adult retina to oxidize glucose carbon

VOL.
5
1959/6

to CO_2. This difference is sharpened by the omission of Ca^{2+} from the medium since it is only in the adult that glucose oxidation is depressed by Ca^{2+}. Thus, in a Ca^{2+}-free medium, glucose oxidation of the adult retina exceeds that of the young retina by a factor of 13 on a dry wt basis. This change occurs principally between the ages of 8 and 20 days (Fig. 3), and most rapidly in the vicinity of 14 or 15 days. The age at which glucose oxidation comes under the influence of Ca^{2+} was not determined accurately, but at 9 days Ca^{2+} had practically no effect, whereas at 14 days the effect

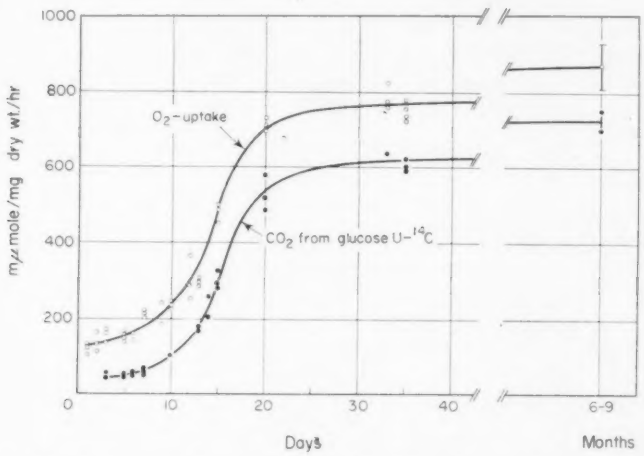


FIG. 3.—Respiration and glucose oxidation of rabbit retina in relation to postnatal age. Medium was calcium free Krebs–Ringer-phosphate buffer containing 20 mM uniformly labelled glucose; incubation was for 65 min.

was as great, on a percentage basis, as that shown by the adult retina. The increase in glucose oxidation during development accounted for practically all of the increase in respiration, so that the oxidation of endogenous substrates (with glucose present) changed little.

The changes with age in glucose oxidation and respiration coincide with the most rapid phase of morphological and functional development of the retina (NOELL, 1958*a*, 1958*b*). At 9 days, the central area, which is most rapidly developing, just begins to function but, at 20 days, function throughout the retina appears to be the same as in the adult except for scotopic capacities.

II. *Pathways of Glucose Oxidation*

Young retina

The participation of the various pathways in the catabolism of glucose was studied by the use of ^{14}C glucose labelled in positions 1, 2, or 6 or uniformly in all positions. Data for the young retina (Table 3) show that the various carbon atoms of glucose are oxidized to CO_2 at very different rates. C-6 oxidation represented only 2–4 per cent of the total glucose carbon oxidized; this result indicates that little glucose was oxidized to completion in the citric acid cycle. C-1 oxidation was also low, but much greater than C-6 oxidation and, since the oxidation of C-1 through

the citric acid cycle cannot exceed that of C-6, the major part of the CO_2 formed from C-1 must have arisen by a route which oxidized glucose asymmetrically, presumably the pentose phosphate path.

By far the largest amount of CO_2 produced from glucose appears to have come from positions 3 and 4. In experiment 31, for instance, only 12 of the 46 mμmoles of

TABLE 3.—CARBON DIOXIDE FORMATION FROM VARIOUS POSITIONS OF GLUCOSE

Each flask contained 0·02 M-glucose labelled uniformly or singly in position 1, 2, or 6. The values are expressed as mμmoles CO_2 formed from the labelled positions of glucose/mg dry wt per hr. Mean data are from experiments performed in the same way as those individually listed; the number of experiments are indicated in parenthesis.

	Young retina		Adult retina	
	Expt. No. 31	Expt. No. 37	Expt. No. 42	Mean
(a) Total CO_2 from glucose-U-^{14}C	46	45	321	320(5)
(b) CO_2 from position 1 (glucose-1-^{14}C)	5·6	4·4	40	46(5)
(c) CO_2 from position 2 (glucose -2-^{14}C)	4·0	3·9	56	52(4)
(d) CO_2 from position 6 (glucose-6-^{14}C)	2·0	0·9 (0·8*)	35	36(4)
(e) Apparent CO_2 from position 3 + 4 (a − b − 2c − d)	30	32	134	134
(f) Deficit between pyruvate decarboxylation and complete oxidation to CO_2 2 × (e − c − d)	48	54	86	92
(g) Glucose carbon in glutamic acid* (glucose-U-^{14}C)	20; 23		42; 46	

* Different experiment(s).

glucose carbon oxidized to CO_2 are accounted for by the oxidation of carbons-1, -2, and -6. If we make the assumption that C-5 oxidation equalled that of C-2 (4 mμmoles), then 30 mμmoles, that is 65 per cent of the CO_2 formed from glucose, arose from carbon atoms 3 and 4 (row (e), Table 3). Theoretically these carbon atoms can be oxidized in the pentose phosphate path, but only at a lower rate than that of the oxidation of C-1 and C-6 by that route. Since the total oxidation of C-1 and C-6 amounted to less than 8 mμmoles, most of the CO_2 derived from positions 3 and 4 was apparently produced by the decarboxylation of pyruvate.

Of the acetyl groups formed in the decarboxylation of pyruvate, few, if any, were

VOL. 5 1959/6

oxidized to completion in the Krebs cycle, as evidenced by the low C-6 oxidation. Most of the carbon atoms of these acetyl groups must have accumulated as intermediates or as end products other than CO_2. It is estimated that approximately 50 mμatoms of glucose-carbon, equal in number to the glucose-carbons that were converted to CO_2, escaped oxidation after the step of pyruvate decarboxylation, on the assumption that the oxidation of carbons -2 and -6 represents exclusively Krebs cycle activity and that C-5 oxidation equals that of C-2. Deviations from these assumptions would increase the value slightly. A substantial fraction (almost 50 per cent) of the missing carbon atoms was found in glutamic acid (Table 3).

Adult retina

The pattern of glucose oxidation in the adult retina (Table 3) differs in several respects from that of the young. All carbon atoms of glucose were converted to CO_2 at a much greater rate than in the young retina, the most striking increase being in C-6 oxidation. Hence, the differences in rate of oxidation among the various carbon atoms of glucose were less significant than in the young retina. C-1 oxidation was higher than that of C-6 but the relative difference was small. It is clear, however, that the average oxidation of C-3 and C-4 (calculated as in the case of the young retina upon the assumption that C-5 oxidation is equal to C-2 oxidation) was higher than the oxidation of the other carbon atoms, particularly that of C-6. This indicates that a large part of the oxidation of C-3 and C-4 proceeded by the decarboxylation of pyruvate. A substantial number of acetyl groups so formed seems to have escaped complete oxidation to CO_2 (row (f), Table 3). As in the young retina, about half of these missing carbon atoms were found in glutamic acid (row (g)).

In both the young and the adult retina, almost all of the lactate produced must have arisen via the Embden–Meyerhof system. Even if the pentose phosphate path were responsible for all of the observed oxidation of C-1 and C-6, which is highly improbable (see below), it would account for less than 15 percent of the lactate formed in the adult and for only 2 percent in the young retina.

III. *Pentose Phosphate Path*

The quantitative data on the oxidation of the various carbon atoms of glucose suggest strongly that the pentose phosphate path operates in the retina under the ordinary condition *in vitro*. In order to obtain information about its capacities, the pattern of glucose oxidation was studied under circumstances which stimulate CO_2 production by this path and/or depress glucose oxidation via the Embden–Meyerhof path. For this purpose we used pyruvate, iodoacetate, and anaerobiosis, separately and in combination. Pyruvate was used because, as first shown by KINOSHITA (1957) for the cornea, it permits the anaerobic operation of the pentose phosphate path by accepting electrons from TPNH.

Since pyruvate added to the medium is an important tool in evaluating the capacities of the pentose phosphate pathway in the retina it is appropriate to list briefly its main effects upon glycolysis and respiration. When unlabelled pyruvate (10 mM) was present in the medium throughout the incubation period, lactic acid formed from glucose was diluted 70 per cent for the young retina and 62 per cent for the adult (Table 4). That this dilution actually occurred in the pyruvate pools is indicated by the recovery measurements listed in Table 5. The sum of the counts in lactate,

pyruvate, and CO_2 were about the same whether pyruvate was added or not, and the counts missing in lactate and CO_2, when pyruvate was present, were almost precisely accounted for by the counts in the pyruvate fraction. It is apparent, furthermore, that pyruvate did not disappear at a rapid rate when glucose was present, and

TABLE 4.—RELATIVE SPECIFIC ACTIVITY OF LACTATE FORMED FROM GLUCOSE-U-^{14}C

The relative specific activity of lactate is expressed as specific activity of lactate carbon ÷ specific activity of glucose carbon. Each flask contained one retina; incubation was for 70 min. Lactate was isolated and counted as described under Methods. The specific activity of glucose did not change significantly during incubation.

Incubation	Young retina	Adult retina
20 mM-Glucose-U-^{14}C	1·00 1·05 1·06	1·00 1·08
20 mM-Glucose-U-^{14}C 10 mM-Pyruvate	0·30 0·29	0·36 0·39

TABLE 5.—ADULT RETINA: RADIOACTIVITY OF LACTATE, CO_2, AND PYRUVATE WITH AND WITHOUT UNLABELLED PYRUVATE PRESENT

One whole retina per flask was incubated in 3 ml medium for 70 min. Each flask contained 60 μmoles glucose (20 mM) uniformly labelled. If present, pyruvate (unlabelled) was added at the beginning of the experiment.

Pyruvate added	Lactate 1	Pyruvate 2	Lactate 3	CO_2 4	Pyruvate 5	Sum (3 + 4 + 5)
	(μmoles at end of expt.)		(counts/min)			
None	9·4	0·23	2290	380	55	2725
30 μmoles	9·3	29·2	850	290	1720	2860

that it did not affect lactic acid production, a fact which is substantiated by the data listed in Tables 6 and 7. Pyruvate (10 mM) increased O_2 uptake slightly in both the young (Table 6) and the adult retina (Table 7) in the presence of glucose but it did not inhibit the respiration of the young retina in the absence of glucose, i.e. it did not elicit the Crabtree effect (Table 6, see values marked E). It diminished the respiratory depression resulting from 2×10^{-3} M-iodoacetate in the adult retina but only from 65 per cent to 40 per cent. In the adult retina pyruvate apparently did not mix readily with that derived metabolically from glucose and used in the citric acid cycle, as will be discussed subsequently.

VOL. 5 1959/6

Young retina (Table 6)

The addition of pyruvate increased the aerobic oxidation of C-1 of glucose while diminishing the oxidation of the other glucose-carbon atoms (cf. columns 1 and 2, Table 6). This diminution, most clearly shown with glucose-U-^{14}C, probably resulted from the dilution of metabolically formed pyruvate, since the radioactivity of lactate was diluted 70 per cent. The preferential increase in C-1 oxidation is presumed to be due to the ability of pyruvate to act as electron acceptor for the pentose phosphate path, and indicates that the availability of electron acceptors is a limiting factor for the operation of this pathway.

The greatest activity of the pentose phosphate path was observed during anaerobiosis (column 3, Table 6). When pyruvate (10 mM) was present in the medium, C-1 oxidation was about twice as high anaerobically as it was aerobically, and represented the bulk of the total glucose oxidation (compare columns 2 and 3). Apparently O_2 exerted an inhibitory influence upon the pentose phosphate path similar to its effect upon glycolysis.

In the presence of pyruvate and O_2, C-1 oxidation was stimulated by iodoacetate while lactic acid production was inhibited 83% (compare columns 2 and 4, Table 6). Possibly, iodoacetate by preventing the glycolytic formation of DPNH facilitates the use of TPNH in the lactic acid dehydrogenase reaction, or it may be that more glucose-6-phosphate becomes available to the pentose phosphate path when glycolysis has been blocked under aerobic conditions.

The activity of the pentose phosphate path in the presence of iodoacetate must be assumed to be greater than would appear from C-1 oxidation alone. With iodoacetate present, the oxidation of C-6 and C-2 must also occur mainly in the pentose phosphate path, since their oxidation in the citric acid cycle should be negligible. That this is indeed the case is indicated by the fact that in the iodoacetate experiment the C-2 oxidation was very low when pyruvate was omitted (column 5, Table 6). Pyruvate actually stimulated C-2 and C-6 oxidation in the presence of iodoacetate (compare columns 4 and 5). It is apparent, therefore, that recycling of the products of phosphogluconate oxidation occurs when iodoacetate has blocked the Embden–Meyerhof path, provided that sufficient electron acceptors are available. Neither the stimulation by iodoacetate nor the inhibition by O_2 of phosphogluconate oxidation were observed to a significant degree when pyruvate was not added to the medium (*cf.* columns 1, 5, and 6). Apparently these effects require that the pentose phosphate path is not limited by a lack of electron acceptors.

Adult retina (Table 7)

As in the case of the young tissue, the existence of the pentose phosphate path was best evident when pyruvate was present under anaerobic conditions (column 3, Table 7). C-1 oxidation was then very high, actually higher than C-6 oxidation aerobically (column 1), and represented 80 per cent of the anaerobic glucose oxidation. Practically no CO_2 was formed from C-2 and C-6 anaerobically (with pyruvate present), evidence that there was no recycling of products of the pentose phosphate path. Either the recycling process was not very active or the high glycolytic capacity of the tissue prevented recycling by trapping the products of the pentose phosphate path (triosephosphate and fructose-6-phosphate) in the form of lactic acid. It is possible to deduce from this finding that C-1 was the only carbon atom of glucose oxidized

TABLE 6.—YOUNG RETINA: EFFECTS OF PYRUVATE, ANAEROBIOSIS AND IODOACETATE ON THE PENTOSE PHOSPHATE PATHWAY

The flasks contained 0·02 M-glucose labelled uniformly or singly in position 1, 2 or 6. For the conditions listed in columns 2, 3 and 4, the medium contained in addition 0·01 M-unlabelled pyruvate. Iodoacetate (columns 4 and 5) was present in the medium at a concentration of 2×10^{-3} M. All values are expressed in mμmoles/mg dry wt per hr. The values listed for O_2 uptake and lactic acid production represent the means of the determinations made on the same retinas as listed individually for the CO_2 formed; the numbers of flasks are given in parenthesis. The four data in columns 1 and 2 which are listed as (E =) represent mean values (n = 3) of simultaneously performed measurements on retinas which were incubated with no glucose present.

		Aerobic (1)		Aerobic + pyruvate (2)		Anaerobic + pyruvate (3)		Aerobic + pyruvate + IAA (4)		Aerobic + IAA (5)		Anaerobic (6)	
Expt.		No. 31	No. 37	No. 31	No. 37	No. 31	No. 37	No. 31	No. 37	No. 31	No. 37	No. 31	No. 37
	O_2 Uptake	161(4) (E = 254)	170(4)	187(4) (E = 258)	176(4)	0(4)	0(2)	126(4)	163(4)	112(4)	165(4)	0(4)	0(2)
	Lactic acid	476(4) (E = 47)	495(4)	437(4) (E = 158)	428(4)	—	1180(2)	116(4)	88(4)	53(4)	49(4)	1050(3)	1215(2)
a	U-^{14}C	46	45	30	29	22·4	19·7	14	13·3	6·4	4·4	4·3	3·6
b	1-^{14}C	5·6	4·4	8·0	7·0	18·2	16·4	10	10·7	3·0	3·3	3·9	3·3
c	6-^{14}C	2·0	0·9	1·9	0·5	—	—	2·7	2·1	1·1	0·7	<0·2	—
d	2-^{14}C	4·0	3·9	3·2	1·6	—	—	1·9	1·4	0·3	0·3	—	—
e	Probable CO_2 production by P.P. path	3·6 b − c	3·5	6·1 b − c	6·5	18·2+ b+	16·4+	14·6 b + c + d	14·2	4·1 b + c	4·0	3·9+ b+	3·3+

VOL. 5 1959/6

BIOL.
5
9/60

TABLE 7.—ADULT RETINA: EFFECTS OF PYRUVATE, ANAEROBIOSIS AND IODOACETATE ON THE PENTOSE PHOSPHATE PATHWAY

Conditions the same as in Table 6.

		Aerobic (1)		Aerobic + pyruvate (2)		Anaerobic + pyruvate (3)		Aerobic + pyruvate + IAA (4)		Aerobic + IAA (5)		Anaerobic (6)
		Expt. 42	Mean	Expt. 42	Mean	Expt. 42	Mean	Expt. 42	Mean	Expt. 42	Mean	Mean
	O_2 Uptake	484(4)	480(17)	465(4)	505(18)	0	0	283(4)	290(8)	165(4)	145(8)	0
	Lactic acid	830(4)	775(17)	820(4)	766(18)	—	1620(2)	127(4)	122(7)	63(4)	—	1470(4)
a	U-^{14}C	321	320(5)	265	253(5)	52	50(2)	22	19(4)	10·1	10·3(2)	4·3(4)
b	1-^{14}C	40	46(5)	57	55(6)	42	40(2)	12	11(3)	3·7	3·8(2)	1·4(4)
c	6-^{14}C	35	36(4)	31	29(6)	0·5	0·55(2)	5	—	2·9	2·8(2)	—
d	2-^{14}C	56	52(3)	44	—	1·4	1·3(2)	1·2	1·5(2)	1·1	1·1(2)	—
e	Probable CO_2 production by P.P. path	5 b − c	10	26 b − c	26	42+ b+	40+	17+ b+ c+	—	3·7+ b+	3·8+	1·4 b+

aerobically via the pentose phosphate path at an appreciable rate in the adult retina although aerobic oxidation of C-2 and C-6 was so much higher than in the young tissue. The anaerobic condition itself should not prevent recycling any more than it prevents C-1 oxidation, except to the extent that glycolysis is higher than it is aerobically and might, therefore, trap more of the products of the first turn of the cycle. Indeed, KINOSHITA (1957) has shown that in the corneal epithelium rapid recycling can be demonstrated anaerobically in the presence of pyruvate. The absence of recycling under these conditions therefore suggests that appreciable recycling does not occur aerobically either, and that the oxidation of C-2 and C-6 observed in the aerobic experiments (columns 1 and 2, Table 7) was effected mainly in the citric acid cycle. If so, almost none of the oxidation of carbon atoms 3, 4, and 5 could have occurred in the pentose phosphate path, since this could happen only after the oxidation of C-2 or C-6 by this route.

Although the oxidation of glucose-carbons 2 and 6 seemed to occur in the adult retina by way of the citric acid cycle, it was only slightly diminished by the addition of unlabelled pyruvate to the medium (compare columns 1 and 2, Table 7); in fact, pyruvate reduced C-6 oxidation by less than 20 per cent. In contrast, pyruvate markedly diluted the pyruvate pool from which lactate was produced, diminishing the radioactivity of lactate by more than 60 per cent (Tables 4 and 5). These findings suggest that pyruvate formed from glucose en route to lactate is not homogenous with that formed en route to the citric acid cycle.*

Since our data indicate that C-6 oxidation of the adult retina is mainly effected by the citric acid cycle, the difference between C-1 oxidation and C-6 oxidation probably represents a good estimate of phosphogluconate decarboxylation. For several reasons the rates of pentose phosphate path activity estimated in this way (Table 7, row e) are considered to be minimal values. It is possible that C-1 oxidation in the pentose phosphate path is followed by the oxidation of C-6 from the same molecule via the citric acid cycle. The latter process, however, should be small, since as pointed out above, the products of the pentose phosphate path would be expected to be trapped as lactate in preference to oxidation in the citric acid cycle. The estimation of the extent of C-1 oxidation in the pentose phosphate path on the basis of the excess over C-6 oxidation, furthermore, involves the assumption that C-1 oxidation in the citric acid cycle is equal to that of C-6, whereas, as pointed out by WOOD (1955), it might actually be less because pyruvate entering the citric acid cycle may not arise equally from the two halves of the glucose molecule. Another phenomenon which could obscure the measurement of the pentose phosphate path would be the extensive operation of the glucuronic acid pathway in which C-6 is oxidized to CO_2 before any other carbon atom (BURNS and KANFER, 1957; MCCORMICK and TOUSTER, 1957). The evidence suggests, however, that this pathway did not make a major contribution to glucose oxidation. C-6 was oxidized apparently more slowly than C-3 or C-4, and its oxidation was greatly diminished by iodoacetate. This is the picture expected if C-6 oxidation occurred mainly by way of the Embden–Meyerhof path and citric acid cycle.

* BLOCH-FRANKENTHAL and RAM, Sept. 1959 Cancer Research **19**: 835, describe a similar finding for ascites tumour cells. Non-labelled pyruvate virtually failed to dilute labelled CO_2 formed from glucose-U-^{14}C. It is implied that this is characteristic of cancer cells in contrast to 'normal tissues'. The authors further describe that pyruvate also failed to dilute the CO_2 arising from the oxidation of lactate-1-^{14}C, though oxidative decarboxylation of pyruvate proceeded simultaneously with that of lactate. They did not measure whether or not pyruvate diluted the lactic acid formed from glucose.

VOL.
5
1959/6

Despite these uncertainties concerning the extent of the activity of the pentose phosphate path in the adult retina it is clear (Table 7) that this path was stimulated by pyruvate. The results suggest, moreover, that with pyruvate present the activity of the pentose phosphate path was greater in the absence of oxygen than in its presence (*cf.* columns 3 and 2, Table 7), as was shown also for the young retina. In contrast to the findings with the young retina, however, the C-1 oxidation in the pentose phosphate path in the presence of pyruvate was decreased by iodoacetate (*cf.* columns 4 and 2). The difference may be related to the fact that in the adult retina iodoacetate

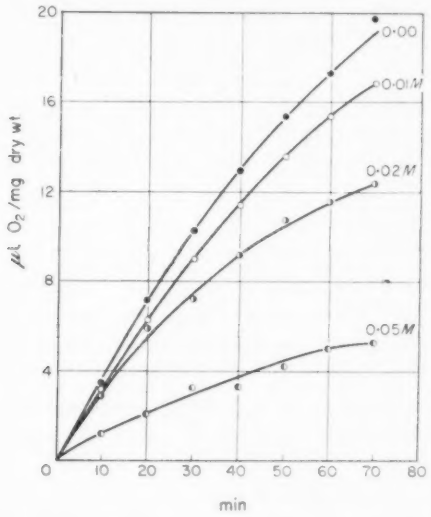


FIG. 4.—Adult retina: Effect of malonate. Medium: 3·0 ml calcium-free Krebs–Ringer-phosphate buffer containing 20 mM-glucose. Concentrations of malonate are shown on the right.

inhibited not only lactate formation but also respiration, even with pyruvate present so that the production of ATP for the phosphorylation of glucose may have been limiting for the operation of the pentose phosphate pathway under these conditions.

Effect of malonate upon retinal respiration

The above results indicate that most of the glucose oxidation of the adult retina proceeds via the citric acid cycle. Since glucose oxidation was found to be responsible for a major fraction of the respiration, one would expect respiration to be inhibited by malonate. Indeed, malonate inhibited respiration (Fig. 4) although high concentrations or long periods of incubation were required for a marked effect.

DISCUSSION

General considerations. Vertebrate retinas resemble one another in general structure but differ among species in many details, such as cell number per unit area, thickness of the various retinal layers, ratio of cones to rods and fine structure of the photoreceptors. There are differences also in the nutritional supply of the retina. The rabbit retina, for instance, is essentially avascular, and in apparent relation

thereto its inner layers differ from those of monkey retina in the concentrations of lactic dehydrogenase and of enzymes of the citric acid cycle (LOWRY, ROBERTS and LEWIS, 1956). The effectiveness of anoxia and iodoacetate on retinal function also show great species-dependent differences (NOELL, 1951). It is, therefore, not surprising that the metabolic capacity of the tissue *in vitro* differs among species. For instance, O_2 uptake measured in the present study was lower than that of rat retina (LASER, 1937; CRAIG and BEECHER, 1943) but higher than reported for ox retina (HOPKINSON and KERLY, 1959). In our experiments, iodoacetate at a concentration that just blocked glycolysis inhibited respiration by 60 per cent in contrast to experiments on the ox retina in which a significant respiratory inhibition by iodoacetate was not observed in Krebs–Ringer-phosphate (HOPKINSON and KERLY, 1959). Perhaps the ox retina oxidizes very little glucose in a phosphate-buffered medium (see also FUTTERMAN and KINOSHITA, 1959), whereas in the rabbit retina the oxidation of glucose to CO_2 accounts for almost 70 per cent of the respiration in this medium. The adult ox retina also shows a distinct Crabtree effect in phosphate buffer (HOPKINSON and KERLY, 1959) whereas in the rabbit the Crabtree effect of the adult tissue is at best slight although very evident with the young tissue.

Retinal glucose catabolism must be considered also in relation to the incubation medium used. Respiration and glycolysis are reported to be much higher in a bicarbonate-buffered medium (CRAIG and BEECHER, 1943; HOPKINSON and KERLY, 1959) than in the phosphate-buffered medium used in the present study. Since the adult rabbit retina respires even in Krebs–Ringer-phosphate more rapidly than it probably does *in vivo* (see below), it is evident that neither medium simulates the *in vivo* condition and that the *in vitro* experiments merely define certain properties of the tissue. The significance of these properties in terms of *in vivo* metabolism will probably be determined only by actual *in vivo* measurements. It was therefore decided to restrict the present study to a single medium simple enough to permit extensive investigation on the metabolic fate of glucose. The medium used, however, did not provide information on the possible influence of CO_2 on the metabolic processes studied.

Retina vs. brain. Although the retina is part of the brain, its metabolism *in vitro* differs in many respects from that of ordinary brain tissue. All mammalian retinas so far examined produce lactic acid aerobically at a much higher rate than brain slices. Moreover, the contribution made by glucose oxidation to the respiration of the adult rabbit retina is higher than reported for brain (for comparable brain data see SUTHERLAND, BURBRIDGE and ELLIOTT, 1955; WENNER and WEINHOUSE, 1956; DIPIETRO and WEINHOUSE, 1959). The rabbit retina differs from cerebral cortex also in its response to changes in the potassium concentration of the medium. Retinal respiration and glucose oxidation were distinctly depressed by either a high K^+ concentration or by K^+-lack, whereas lactic acid production was practically unaffected. In contrast, respiration and aerobic glycolysis of cortex slices are not affected by the omission of potassium and are stimulated by high K^+ concentrations (ASHFORD and DIXON, 1935; GORE and MCILWAIN, 1952; TOWER, 1958). The marked stimulation of retinal respiration by Ca^{2+} lack, on the other hand, is similar in magnitude to that reported for cerebral cortex. In the rabbit retina this stimulation appears to be due to a stimulation of glucose oxidation but this has not been shown for other nervous tissues.

VOL.
5
1959/6

Another difference between the metabolism of the adult retina and that of brain concerns the activities *in vitro* as compared to those *in vivo*. The rates at which cerebral tissue slices respire *in vitro* are only about half of those computed for respiration *in vivo* (see McIlwain, 1955). For the rabbit retina the reverse situation seems to exist as indicated by a consideration of retinal oxygen supply. Since this retina is exclusively supplied by the choroidal capillaries behind the retina it is easy to compute the pressure gradient of O_2 needed for the maintenance of respiration over the whole thickness of the retina which represents a tissue slice 130 μ thick nourished from only one side (see Warburg, 1930, p. 79 equation No. 3). If it is assumed that the mean O_2 pressure of the choroidal blood is 60 mm Hg and that the respiratory rate is the same for all layers of the retina, then a zone of anoxia at the inner retinal surface would result if respiration exceeded a Q_{O_2} of $-4{\cdot}2$, which is less than half the rate measured in our experiments. This computation is not affected significantly by the more realistic assumption that O_2 uptake differs for the different retinal layers. In fact, if respiration is assumed to parallel the local concentration of malic dehydrogenase (Lowry, Roberts and Lewis, 1956), an essentially similar result is obtained (Janney and Noell, unpublished). It is unlikely that O_2 is the limiting factor for the *in vivo* respiration because the O_2 pressure measured *in vivo* polarographically by platinum electrode at the inner surface of the rabbit retina, or within the vitreal body close to the retina, is distinctly above zero (Noell, unpublished). It is more likely that the high *in vitro* rate of respiration is conditioned by changes in the fine structure of the retinal elements due to the artificial environment. As isolated mitochondria are less active the better their structure is preserved (Ziegler and Linnane, 1958) so also the rabbit retina *in vivo* might be considerably more restrained in its respiratory activity than *in vitro*. The same may very well apply to the glycolytic capacities.

Young vs. adult retina. The greatest difference between the young and adult retina was in the rates of glucose oxidation by the citric acid cycle and by the pentose phosphate pathway. The capacities of both pathways were much higher in the adult tissue. However, since the difference in glucose oxidation was most marked for C-6, and since C-6 oxidation in the adult retina appeared to occur mainly in the citric acid cycle, it would seem that the most striking developmental change revealed by these studies is in the oxidation of glucose in the citric acid cycle. The lack of C-6 oxidation in the young retina does not necessarily mean that no citric acid cycle activity exists at this age. About 65 per cent of the CO_2 produced is not derived from glucose and the most probable source is the oxidation of non-carbohydrate materials in the citric acid cycle. The fact that a considerable amount of glucose-carbon was incorporated into glutamate is strong evidence for the activity of at least part of the citric acid cycle.

The quantitative differences in metabolism between the young and adult retina measured in the present study agree with the findings of Schimke (1959) that the concentrations of glucose-6-phosphate dehydrogenase and malic dehydrogenase in rabbit retina are considerably higher at 26 days than at birth, whereas the lactic dehydrogenase content is changed only slightly. If compared on a dry weight basis, the maximal capacity for respiration as measured in our experiments increased more with age than malic dehydrogenase and that for the pentose phosphate pathway increased slightly less than glucose-6-phosphate dehydrogenase. Maximal lactic acid production in our experiments was only slightly higher (25 per cent) in the adult

than in the young retina as compared to a slight decrease in lactic dehydrogenase. In contrast, the anaerobic glycolysis of rat retina measured in a bicarbonate-buffered medium more than doubled during post-natal development (GRAYMORE, 1959).

The respiratory increase in the rabbit retina is related closely in time with the differentiation of the visual cells and with the appearance and maturation of visual function as measured by the electroretinogram (NOELL, 1958). However, it is impossible to decide how much of the respiratory increase might reflect the simultaneously occurring differentiation of the visual cells and the spectacular development of the rods and cones which contain, in their inner segment, the mitochondria of the visual cells.

Crabtree effect. The inhibition of the respiration of the young retina by glucose is of interest with regard to a similar effect in tumour tissues. When Crabtree discovered this effect with tumour slices in 1929, he concluded tentatively that "glycolytic activity exerts a significant checking effect on the capacity for respiration of tumour tissue". In recent years, intensive studies of the Crabtree effect of ascites tumour cells have led to the view that more than one mechanism may be involved, but that to a great extent the inhibition is due to a competitive interaction between enzymes of the glycolytic system and those of respiration for some cofactor such as inorganic phosphate or adenine nucleotides (BRIN and MCKEE, 1956; WU and RACKER, 1957, 1959). The only non-neoplastic tissues for which the Crabtree effect has been shown are the retina and the leukocytes (MARTIN, MCKINNEY and GREEN, 1955). The occurrence of the effect in these tissues, which share with tumour tissue the property of high aerobic glycolysis, supports the competition hypothesis. However, the Crabtree effect displayed by the adult rabbit retina is at best very slight. This may be related to the fact, revealed by electron microscopy (DEROBERTIS and FRANCHI, 1956) and histochemistry (LOWRY, ROBERTS and LEWIS, 1956), that there is a considerable degree of separation of the elements of glycolysis and respiration in the adult retina, particularly in the visual cells (NOELL, 1958*a*). It is also possible that the Crabtree effect cannot become evident in a tissue in which respiration is due mainly to glucose oxidation.

Pentose phosphate pathway; possible significance of glucose catabolism in retinal function. In the rabbit retina, most of the CO_2 produced from glucose under ordinary experimental conditions was formed by way of the Embden–Meyerhof path and citric acid cycle. However, the addition of pyruvate to the medium under anaerobic condition revealed a high capacity for glucose oxidation via the pentose phosphate path in both the young and adult retina. The rate of C-1 oxidation under these conditions is very similar to that reported by KINOSHITA (1957) for corneal epithelium, a tissue in which the pentose phosphate path appears to be the major route of glucose oxidation. Since the first report on the pyruvate-stimulated, perferential C-1 oxidation by the retina (COHEN and NOELL, 1958) FUTTERMAN and KINOSHITA (1959) independently showed anaerobic C-1 oxidation to be stimulated by pyruvate in the ox retina; VON HOLT *et al.* (1959) reported a small preponderence of C-1 oxidation over that of C-6 under ordinary aerobic conditions in the ox retina.

Although the capacity of the pentose phosphate path demonstrated in this way is almost half the aerobic glucose oxidation in the young retina and is as high as C-6 oxidation by the citric acid cycle in the adult retina, it is very small in comparison with the high rate of lactate formation by the Embden–Meyerhof system. However,

VOL.
5
1959/6

the latter comparison is probably not the important one since the two pathways seem to have different functions, in that the Embden–Meyerhof path is a source of phosphate-bond energy, whereas the primary function of the pentose phosphate path is apparently to provide TPNH for reductive reactions, and intermediates for biosynthesis (KREBS, 1954; KAPLAN, SWARTZ, FRECH and CIOTTI, 1956).

The chief limiting factor for the pentose phosphate pathway appears to be the rate of oxidation of TPNH, and oxygen does not seem to be effective for this process. That pyruvate can furnish considerable oxidizing capacity *in vitro* was first indicated by the experiments of CRANE and BALL (1951) with ox retina. They obtained indirect evidence that CO_2-fixation via the malic enzyme was coupled to TPNH formation in the glucose-6-phosphate dehydrogenase reaction. However, since the present experiments were performed with a CO_2-free medium this mechanism is probably not responsible for the observed effect of pyruvate. Another possible reaction by which pyruvate may oxidize TPNH is that catalysed by lactic dehydrogenase. Although this enzyme is much more active with DPNH than with TPNH as cofactor, it is apparently able to catalyse the oxidation of TPNH at a rate commensurate with decarboxylation via the phosphogluconate oxidation pathway as observed for the cornea (KINOSHITA, 1957) and recently for the retina as well (KINOSHITA and FUTTERMAN, 1959). In liver, too, a similar mechanism of TPNH oxidation seems to exist (NAVAZIO, ERNSTER and ERNSTER, 1957). Our measurements of anaerobic C-1 oxidation with pyruvate present may not, however, indicate the maximal capacity of C-1 oxidation in the pentose phosphate path. Thus in ascites tumour cells methylene blue, menadione and phenazine methosulphate are more effective stimulants of C-1 oxidation than is pyruvate (WENNER, HACKNEY and MOLITERNO, 1958).* Nevertheless, the use of pyruvate in our study provided evidence on limiting factors of the pentose phosphate path other than those imposed by the lack of electron acceptors.

A controlling mechanism seemed to exist between respiration and the pentose phosphate path as evidenced by the inhibition of C-1 oxidation by O_2 in young and adult retina. This inhibition is analogous to the Pasteur effect, but a suitable explanation is not at hand. In the young retina, furthermore, evidence was obtained from the effect of iodoacetate on the pyruvate-stimulated C-1 oxidation suggesting a competition between glycolysis and the pentose phosphate path in reactions involving glucose-6-phosphate or the reduction of pyruvate. A competition between the two pathways, though involving other reactions, has also been demonstrated recently by COURI and RACKER (1959) in a system reconstructed from enzymes of the pentose-phosphate path.

While pyruvate can provide the oxidizing capacity *in vitro*, the nature of the electron acceptors *in vivo* undoubtedly depends on the kind of tissue. To a great extent, especially in a differentiating tissue like the young retina, the electron acceptors are probably provided by the substrates of the synthetic reactions of cell maintenance and growth, many of which specifically require TPNH. The adult retina, however, displays a much higher capacity of the pentose phosphate path than does the young, and its dependence upon a metabolite of another pathway would make it well suited for a possible role in retinal excitation. For example, the system could act as a

* Recent experiments showed that with the young and adult retina, too, phenazine methosulphate is much more effective than pyruvate in stimulating the preferential oxidation of glucose C-1. With 2×10^{-4} M-phenazine methosulphate the preferential oxidation of C-1 in the adult retina was 6 times greater than that observed with added pyruvate.

switch for the interconversion of TPN and TPNH or for the formation of metabolites of the pentose phosphate path. Such a switch could be operated by electron acceptors (e.g. pyruvate) formed in other metabolic paths.

The possibility that the pentose phosphate path has a role in visual function is suggested by the distribution of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase in rabbit retina. LOWRY (see NOELL, 1958*a*) has found that these enzymes are highly concentrated in the inner segments and in the region of the visual cell synapses, sites which are undoubtedly involved in visual function.

One might speculate as to whether or not the high capacity for glucose oxidation via the Krebs cycle in the adult retina is also closely related to visual function. In the visual cells of the rabbit the mitochondria are confined to the inner segments of the rods and cones, in proximity to the outer segments, i.e. near the site of the primary events in vision. The outer segment appears to have solid-state properties, and it is tempting to consider that visual excitation may involve a rather direct communication by a metabolic chain and electron transport between the outer segment and the mitochondria of the inner segment. But whatever the mechanism of the transmission of 'excitation' in these parts of the visual cell may be, it is of heuristic value at least to assume a specific role of the mitochondria in this process, as was first suggested by SJÖSTRAND (1953).

The striking effects of intravenous iodoacetate on the electroretinogram and on the viability of the visual cells (NOELL, 1951, 1952) suggest that function and maintenance of the mammalian visual cells are extraordinarily dependent upon glycolysis. Since the high effectiveness of a partial inhibition of the Embden–Meyerhof pathway contrasts with the relatively low effectiveness of complete anoxia on retinal function (NOELL, 1953) it is assumed that the Embden–Meyerhof pathway itself provides essential energy. This is well supported by the electron microscopic finding that large parts of the visual cell, including in the rabbit the synaptic ending, are free from mitochondria.

SUMMARY

Retinal respiration and glycolysis, and the metabolic fate of glucose labelled with ^{14}C uniformly or singly in positions 1, 2 and 6 were studied *in vitro* mainly at two ages: when the retina was fully developed and when it was not yet functioning at 5–7 days after birth.

The respiration of the *young* retina was low and was inhibited by glucose (Crabtree effect). Glucose oxidation contributed to the respiration of the young retina only to a small degree and about 65 per cent of the respiration appeared to be due to the oxidation of endogenous non-carbohydrate material when glucose was supplied. In contrast, glucose was the sole source of lactate carbon. Aerobic lactic acid production was about ten times higher than CO_2 production from glucose. The bulk of the CO_2 from glucose appeared to come from positions 3 and 4 at this age. About as many glucose carbons as were converted to CO_2 seemed to escape oxidation after pyruvate decarboxylation; a substantial fraction of the missing carbon atoms was found in glutamic acid.

On a dry weight basis, all metabolic activities of the *adult* retina were higher than those of the young, and the increases occurred principally between the ages of 10 and 20 days, in close correlation with visual cell differentiation and with the

appearance and maturation of retinal function. Anaerobic lactic acid production changed little with age but the oxidation of glucose in the citric acid cycle increased severalfold. Glucose oxidation of the adult tissue accounted for the major portion of respiration and at most only 30 per cent of the oxygen uptake appeared to be due to the oxidation of noncarbohydrate material. A slight inhibition of respiration by glucose was observed but only in comparison to the initial period of respiration in a substrate-free medium. As in the young retina, about half of the carbon atoms which seemed to escape oxidation within the citric acid cycle were found to be incorporated into glutamic acid. In contrast to the young retina, a complete inhibition of the Embden–Meyerhof pathway by iodoacetate was associated with a 60 per cent depression of respiration. Omission of Ca^{2+} from the medium stimulated the respiration of the adult tissue markedly but was ineffective in the case of the young retina.

The capacities for glucose oxidation by the pentose phosphate pathway were studied under conditions which stimulate CO_2 production by this path and/or depress glucose oxidation via the Embden–Meyerhof system. The pentose phosphate path seemed to operate at both ages under ordinary *in vitro* conditions but was severely restricted by the availability of electron acceptors. This limitation was overcome by the addition of pyruvate. With pyruvate present the greatest activity of the pentose phosphate pathway was observed during anaerobiosis. The capacity for C-1 oxidation demonstrated in this way was almost half the total aerobic glucose oxidation under ordinary conditions in the young retina and was as high as C-6 oxidation by the citric acid cycle in the adult retina. The capacity for glucose oxidation by the pentose phosphate pathway as measured anaerobically with pyruvate present was about twice as high in the adult retina as in the young. At both ages it was small in comparison to the high rate of lactate production by the Embden–Meyerhof system. In the young retina glucose oxidation by the pentose phosphate pathway was stimulated by iodoacetate if a sufficiency of electron acceptors was provided.

Addition of unlabelled pyruvate to the medium readily diluted the radioactivity of lactate formed from labelled glucose but diminished only slightly the oxidation of glucose carbon by way of the citric acid cycle.

The possible significance of some of the metabolic properties of the retina was discussed in relation to problems of retinal function and in comparison to other brain tissue.

Acknowledgements.—The authors are indebted to Miss ELIZABETH CARTER for excellent technical assistance at all phases of this study and to Miss A. BUCZKOWSKI for devoted help in preparing the manuscript.

REFERENCES

ASHFORD C. A. and DIXON K. C. (1935) *Biochem. J.* **29,** 157.
BRIN M. and MCKEE R. W. (1956) *Cancer Res.* **16,** 364.
BURNS J. J. and KANFER J. (1957) *J. Amer. chem. Soc.* **79,** 3604.
CALVIN M., HEIDELBERGER C., REID J. C., TOLBERT B. M. and YANKWICH P. F. (1949) *Isotopic Carbon* p. 93. Wiley, New York.
CHANCE B. and HESS B. (1956) *Ann. N.Y. Acad. Sci.* **63,** 1008.
COHEN L. H. (1957) *Fed. Proc.* **16,** 165.
COHEN L. H. and NOELL W. K. (1958) *The Physiologist* **1,** 10.
COHEN L. H. and NOELL W. K. (1959) *Fed. Proc.* **18,** 28.
COURI D. and RACKER E. (1959) *Arch. Biochem. Biophys.* **83,** 195.
CRABTREE H. G. (1929) *Biochem. J.* **23,** 536.

CRAIG F. N. and BEECHER H. K. (1943) *J. gen. Physiol.* **26,** 473.
CRANE R. K. and BALL E. G. (1951*a*) *J. biol. Chem.* **188,** 819.
CRANE R. K. and BALL E. G. (1951*b*) *J. biol. Chem.* **189,** 269.
DEROBERTIS E. (1956) *J. biophys. biochem. Cytol.* **2,** 319.
DEROBERTIS E. and FRANCHI C. M. (1956) *J. biophys. biochem. Cytol.* **2,** 307.
DEROBERTIS E. and LASANSKY A. (1958) *J. biophys. biochem. Cytol.* **4,** 743.
DIPIETRO D. and WEINHOUSE S. (1959) *Arch. Biochem. Biophys.* **80,** 268.
DIXON M. (1951) *Manometric Methods.* Cambridge University Press.
ELENIUS V. (1958) *Acta physiol. scand.* **44,** Suppl. 150.
FIESER L. (1950) *Experiments in Organic Chemistry* (2nd Ed.) p. 393. Heath, Boston.
FUTTERMAN S. and KINOSHITA J. (1959) *J. biol. Chem.* **234,** 723.
GORE M. B. and MCILWAIN H. (1952) *J. Physiol.* **117,** 417.
GRAYMORE C. (1959) *Brit. J. Ophthal.* **43,** 34.
HOPKINSON L. and KERLY M. (1959) *Biochem. J.* **72,** 22.
HORN H. D. and BRUN F. H. (1956) *Biochem. biophys. acta* **21,** 379
KAPLAN N. O., SWARTZ M. N., FRECH M. E. and CIOTTI M. M. (1956) *Proc. Nat. Acad. Sci., Wash.* **42,** 481.
KINOSHITA J. (1957) *J. biol. Chem.* **228,** 247.
KINOSHITA J. and FUTTERMAN S. (1959) *Fed. Proc.* **18,** 260.
KREBS H. A. (1954) *Johns Hopk. Hosp. Bull.* **95,** 19.
KUN E., TALALAY P. and WILLIAMS-ASHMAN H. G. (1951) *Cancer Res.* **11,** 855.
LASANSKY A. and DEROBERTIS E. (1959) *J. biophys. biochem. Cytol.* **5,** 245.
LASER H. (1937) *Biochem. J.* **31,** 1671.
LINDBERG B. and WICKBERG B. (1954) *Acta chem. scand.* **8,** 569.
LOWRY O. H., ROBERTS N. R. and LEWIS G. (1956) *J. biol. Chem.* **220,** 879.
MARTIN S. P., MCKINNEY G. R. and GREEN R. (1955) *Ann. N.Y. Acad. Sci.* **56,** 996.
MCCORMICK D. B. and TOUSTER O. (1957) *J. biol. Chem.* **229,** 451.
MCILWAIN H. (1955) *Biochemistry and the Central Nervous System,* p. 236. Churchill. London.
MERRITT H. H. and FREMONT-SMITH F. (1938) *The Cerebrospinal Fluid,* p. 333. Saunders, Philadelphia.
NAVAZIO F., ERNSTER B. B. and ERNSTER L. (1957) *Biochim. biophys. acta* **26,** 416.
NOELL W. K. (1951) *J. cell. comp. Physiol.* **37,** 283.
NOELL W. K. (1952) *J. cell comp. Physiol.* **40,** 25.
NOELL W. K. (1953) *Studies on the Electrophysiology and the Metabolism of the Retina.* U.S.A.F. Air University, School of Aviation Medicine Project No. 21-1201-0004. pp.1-122.
NOELL W. K. (1958*a*) *Arch Ophthal, N.Y.* **60,** 702.
NOELL W. K. (1958*b*) *Ann. N.Y. Acad. Sci.* **74,** 337.
RACKER E. (1956) *Ann. N.Y. Acad. Sci.* **63,** 1017.
SCHIMKE R. T. (1959) *J. biol. Chem.* **234,** 700.
SJÖSTRAND F. S. (1953) *J. cell. comp. Physiol.* **42,** 45.
SUMMERSON W. H. (1939) *J. biol. Chem.* **131,** 579.
SUTHERLAND V. C., BURBRIDGE T. N. and ELLIOTT H. W. (1955) *Amer. J. Physiol.* **180,** 195.
TOWER D. B. (1958) *J. Neurochem.* **3,** 185.
VON HOLT C. H., LUTH I., VON HOLT L. and VOLKER I. (1959) *Biochem. Z.* **331,** 239.
WARBURG O. (1927) *Biochem. Z.* **184,** 484.
WARBURG O. (1930) *The Metabolism of Tumours.* Constable, London. p. 79.
WARBURG O., POSENER K. and NEGELEIN E. (1924) *Biochem. Z.* **152,** 309.
WENNER C. E., HACKNEY J. H. and MOLITERNO F. (1958) *Cancer Res.* **18,** 1105.
WENNER C. E. and WEINHOUSE S. (1956) *J. biol. Chem.* **222,** 399.
WOOD H. G. (1955) *Physiol. Rev.* **35,** 841.
WU R. and RACKER E. (1957) *Fed. Proc.* **16,** 274.
WU R. and RACKER E. (1959) *J. biol. Chem.* **234,** 1036.
ZIEGLER D. M. and LINNANE A. W. (1958) *Biochem. biophys. acta* **30,** 33.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 277 to 282. Pergamon Press Ltd. Printed in Northern Ireland

PROTEINASE ACTIVITY OF PERIPHERAL NERVES DURING WALLERIAN DEGENERATION*

G. PORCELLATI and B. CURTI
Department of Biochemistry, University of Perugia, Perugia, Italy

(*Received* 12 *October* 1959)

IN A previous study on the content of free amino acids in the hen sciatic nerve (PORCELLATI and THOMPSON, 1957) it was shown that during Wallerian degeneration after section a considerable increase in the levels of several amino acids occurred. This was attributed to an acceleration of the proteolytic processes. The maximal values were reached about 15 days after the section: serine and threonine showed the largest percentage increase.

The purpose of the present investigation was to investigate the activity of 'neutral proteinase' in normal nerves and in nerves undergoing Wallerian degeneration. The 'neutral proteinase', recently discovered in brain tissue by ANSELL and RICHTER (1954), is an enzymic system acting at a good rate on tissue proteins at pH 7·4. We have investigated the neutral proteinase activity in sciatic nerves before and at different times after the section.

EXPERIMENTAL

Homogenates and incubation mixtures. Fully grown hens of mixed breed, weighing about 2 kg, were used throughout. In transection experiments the right sciatic nerve of the animals was cut under buthalitone sodium (Baytinal; Bayer) anaesthesia, as described by PORCELLATI and THOMPSON (1957), care being taken to prevent any re-innervation of the distal stump. After 4, 8, 15, 21 and 28 days the animals were killed, the corresponding segments of the distal transected nerve and of the intact opposite nerve (control) were removed, immediately frozen in crushed ice, and left in a deep-freeze. The control and experimental tissues were treated separately in all the procedures described below.

The tissue was thawed, cleaned of adherent fatty and connective tissue, weighed and ground in a small mortar. After dilution to a final concentration of 1:4 (w/v) with ice-cold, Ca^{2+} free, Krebs phosphate–saline solution buffered with 0·1 M-phosphate buffer pH 7·4, the suspension was finely homogenized and immediately incubated under nitrogen in Warburg vessels for 1 hr at 37°. The incubation was started about 35–40 min after the death of the animal. The quantities of sciatic nerve in each incubation vessel ranged from 280 to 330 mg of fresh tissue. In addition, some experiments were done using cell-free homogenates of brain and spinal cord from the same animals.

Determinations. The enzymic activity was stopped by the addition of 2 ml of ice-cold 20% trichloroacetic acid. A further 1 ml of the buffered phosphate-saline solution described above was added to give a final volume of 6 ml, and the suspension was filtered in the cold. NH_2-N was determined on portions of the filtrate by a modified Moore and Stein ninhydrin procedure (JACOBS, 1956), after conversion of glutamine to pyrrolidone-carboxylic acid and ammonia by heating (ANSELL and RICHTER, 1954). Standard glutamic acid was used for comparison. Ammonia was determined separately by the procedure of PARNAS and HELLER (1925) and all the results were corrected for free ammonia. Peptide estimation was carried out on a further portion of the filtrate by hydrolysis with 6 N-HCl (final concentration) for 36 hr in a sealed tube at 100°. In this instance also a correction was made for the colour due to free ammonia. The protein content of each incubation vessel was determined as described by WARBURG (1948).

Chromatography. Paper chromatographic analysis of the amino acids was carried out by the

* A preliminary account of this work has been given at the IV International Congress of Biochemistry, Vienna, 1958.

method described by PORCELLATI and THOMPSON (1957) on portions of the TCA filtrates corresponding to 10–20 mg of fresh tissue. These experiments were performed to confirm the chemical determinations and to compare the NH_2-N production with the levels of individual amino acids after incubation. Desalting of filtrates was found unnecessary for paper chromatography.

Calculation of the enzyme activity. The enzymic activity was calculated by determining the NH_2-N production after 1 hr of incubation at 37°. The results are expressed either as μmoles NH_2-N /mg wet weight per hr or as μmoles NH_2-N/mg dry weight per hr. In the former instance in order to obtain a true estimation of the enzyme activity, it was necessary to correct for the increase in the water content (oedema) of the nerve which occurs after section (JOHNSON, MCNABB and ROSSITER,

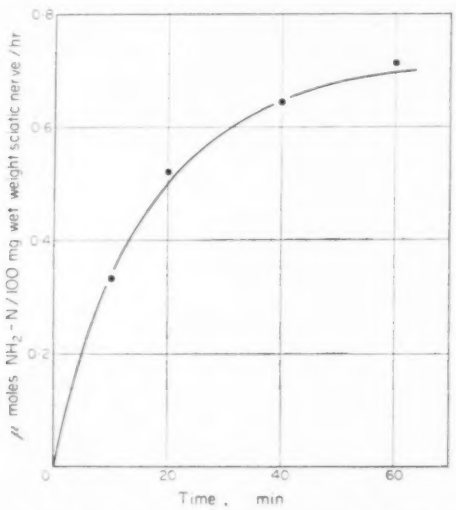


FIG. 1.—Proteinase activity of normal hen sciatic nerve incubated at 37°, pH 7·4. Mean values of 15 experiments.

1949; CAVANAGH, THOMPSON and WEBSTER, 1954). This was done in each experiment by multiplying the activities expressed as μmoles NH_2-N/mg wet weight degenerating nerve per hr by the ratio of the wet weight of the degenerating nerve to the wet weight of a similar length of normal nerve (from the opposite side). This is equivalent to expressing the activities as μmoles NH_2-N/mg wet weight of control nerve per hr.

RESULTS

Intact nerves. The proteinase activity at pH 7·4 of normal hen sciatic nerves, at different intervals of time are shown in Fig. 1. In 15 experiments the proteinase activity after 1 hr incubation ranged from 0·36 to 1·22 μmoles NH_2-N/100 mg sciatic nerve per hr, with a mean value of 0·708 (S.E.M. $\pm$ 0·050). The system is similar to that found by ANSELL and RICHTER (1954) in rat brain and it can be detected only if tested shortly after death. Proteinase activity was also found in hen spinal cord and brain, with a mean value of 0·585 and 0·928 μmole NH_2-N/100 mg fresh tissue per hr respectively.

Paper chromatograms of the same incubation filtrates (see Fig. 3, 1) showed that the concentrations of all the detectable amino acids (aspartic and glutamic acids, serine, glycine, alanine and threonine) increased after incubation. Moreover, the determination of peptide-bond-nitrogen showed that this nitrogen fraction did not change during the experiment (Table 1).

VOL. 5 1959/6

Degenerated nerves. The protein breakdown in sciatic nerves at 4, 8, 15, 21 and 28 days after transection is shown in Fig. 2.

It appears that there was a slight decrease of proteinase activity during the first days after transection, but at the 8th and 15th days there was an increased activity of about 25 and 130 per cent respectively, when calculated at the end of the incubation.

TABLE 1.—THE NH_2-N FORMATION COMPARED WITH THE PEPTIDE-BOND NITROGEN CONTENT IN HEN SCIATIC NERVES

(Incubation at 37°, pH 7·4, gas phase N_2. Mean values of 15 experiments expressed as μmoles/100 mg wet weight tissue)

Incubation time (min)	NH_2-N formed	Δ	Peptide-bond N	Δ
0	0	—	0·56	—
60	0·70	0·70	0·58	0·02

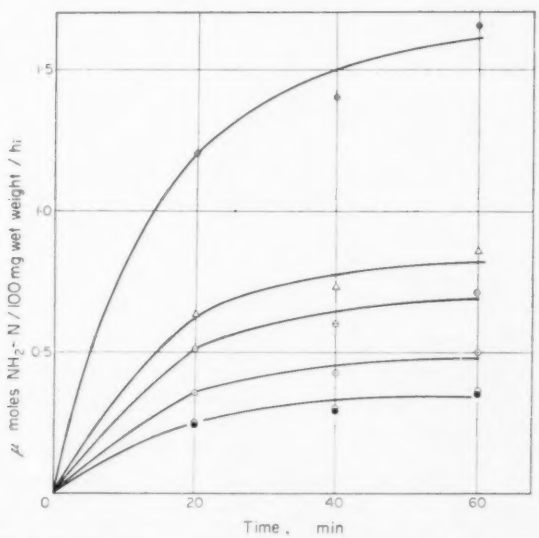


FIG. 2.—Proteinase activity of degenerated sciatic nerve incubated at 37°, pH 7·4 for 60 min. Mean values at 20, 40 and 60 min of incubation of 4 experiments for each day of degeneration. ⊖—⊖—⊖—⊖—⊖ normal; ○—○—○—○—○ 4th day after transection; △—△—△—△—△ 8th day; ⊕—⊕—⊕—⊕—⊕ 15th day; ○—○—○—○—○ 21st day; ●—●—●—●—● 28th day.

At the 21st and 28th days the values were about 50 per cent lower than that of the controls.

The values of NH_2-N liberated after incubation for 1 hr expressed in terms of either wet or dry weight of tissue (Table 2) have been compared. There was no significant difference between the two methods for determining the enzyme activity when this was compared with the corresponding normal values of the controls.

Paper chromatography (Fig. 3, 2 and 3) showed that after incubation of the degenerating nerves an increase in the concentration of several amino acids occurred.

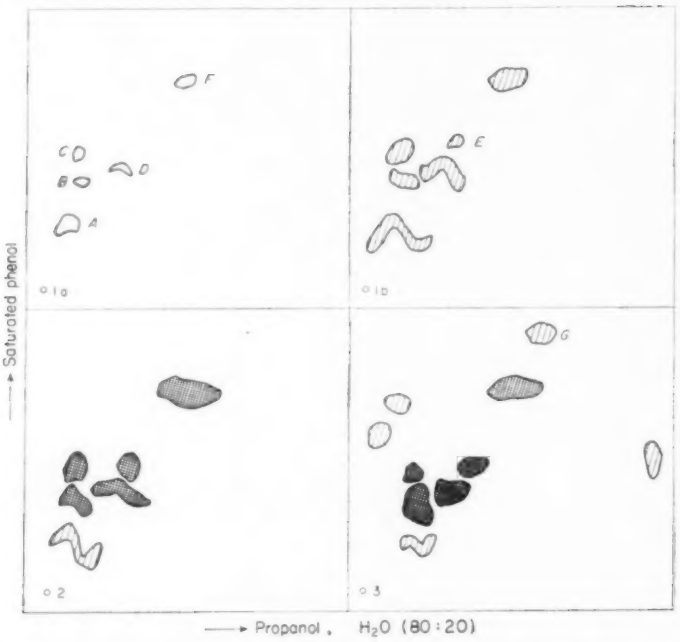


FIG. 3.—Chromatograms of incubation filtrates of hen-sciatic-nerve tissues. (1a) control, zero time; (1b) control, incubated; (2) 8 days of degeneration, incubated; (3) 15 days of degeneration, incubated. A—aspartic acid; B—serine; C—glycine; D—glutamic acid; E—threonine; F—alanine; G—valine.

TABLE 2.—THE PROTEINASE ACTIVITY OF HEN SCIATIC NERVES DURING DEMYELINATION. (Hen sciatic nerve preparation incubated for 60 min at 37° in Krebs phosphate-saline solution, pH 7·4, gas phase N_2. Mean of 4 experiments for each time after section, except for normal nerves where the mean is of 15 experiments)

Experiments	μmoles NH_2-N/100 mg per hr, referred to			
	dry weight	Δ	wet weight	Δ
Normal nerve	1·94	—	0·71	—
4th day after section	0·81	−1·13	0·50	−0·21
8th day after section	2·05	+0·11	0·86	+0·15
15th day after section	4·41	+2·47	1·66	+0·95
21st day after section	0·88	−1·06	0·36	−0·35
28th day after section	1·23	−0·71	0·35	−0·36

The greatest increase among the detectable amino acids was shown by serine, threonine and glutamic acid. At the 8th day of degeneration the increase in the concentration of amino acids was higher than that of the control, and for nerves at the 15th day after transection this increase was even greater (see Fig. 3). Some other, faint, spots appeared in the chromatograms of nerves at the 15th day of degeneration. These have not been identified so far.

The values for the formation of NH_2-N by intact nerves differed considerably

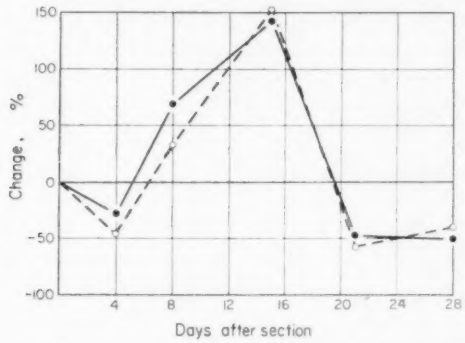


FIG. 4.—Proteinase activity of hen sciatic nerves at intervals after sectioning. ●—●—●—● wet weight nerve; ○—○—○—○ dry weight nerve. Each point is the mean of 4 experiments.

TABLE 3.—THE PROTEIN CONTENT OF HEN SCIATIC NERVES DURING WALLERIAN DEGENERATION

(Mean values of 4 experiments for each day of degeneration, except for normal nerves where the mean is of 15 experiments. Values in mg protein/g wet weight tissue)

Incubation time (min)	Days of degeneration					
	0	4	8	15	21	28
0	157	153	165	132	128	131
60	155	155	163	126	125	133

from one hen to another (range from 0·36 to 1·22 μmoles/hr). In order to obtain a more correct estimation of the proteinase activity during demyelination, the results have been expressed as the percentage change in the sectioned nerve as compared with the control nerve of the same hen (Fig. 4). The change in proteinase activity during nerve degeneration was then even more apparent. In fact, the maximal values obtained were about 150 per cent higher than the controls.

The protein content of nerves before and after incubation was also investigated. These results are given in Table 3. In the unincubated mixtures, a consistent decrease in the total protein content was found following the transection; it was not possible to detect any further appreciable variations in the same mixtures after incubation for 60 min. This last result was expected, as the relatively low amounts of NH_2-N

formed under these conditions were insufficient to produce a fall in protein detectable by our methods.

DISCUSSION

It has been reported by ANSELL and RICHTER (1954) that fresh brain tissue contains an enzymic system which produces proteolysis on brief incubation at pH 7·4. This system has been called 'neutral proteinase'. The evidence presented in this paper shows: (a) that the system in hen sciatic nerve, spinal cord and brain is similar to that described by ANSELL and RICHTER (1954) and is probably an intracellular proteinase, since the NH_2-N is not derived from peptides: and (b) that there was an increase of this enzymic activity after transection. This increase was evident after 8 and 15 days of degeneration, but fell towards the control values over the following days. These findings explain our previous results (PORCELLATI and THOMPSON, 1957), which showed an increase in the free amino acids during nerve degeneration. We have found, in agreement with previous results (PORCELLATI and THOMPSON, 1957), that the concentrations of serine, threonine and glutamic acid increased more than those of the other amino acids.

The total protein content of the nerve was found to decrease after section, in agreement with the preceding results showing an acceleration of the proteolytic activity.

The reason for the variation of the neutral proteinase activity during nerve degeneration is under investigation.

SUMMARY

To investigate the mechanism of the increase in the amounts of free amino acids found in degenerating peripheral nerves after transection, a study was made of the proteinase activity at pH 7·4 of preparations of hen sciatic nerves after section, as compared with corresponding normal nerve tissue.

In the course of nerve degeneration, neutral proteinase activity increased by the 8th day, and at the 15th day reached a maximal value of 150 per cent. The enzymic activity thereafter decreased and, at the 21st and 28th day of degeneration, was only 50 per cent of the control value.

The increase of free amino acids observed in homogenates of degenerating nerves appears to be due to an acceleration of the proteolytic processes.

Acknowledgements.—This investigation was supported in part by a grant from the Consiglio Nazionale delle Ricerche, Rome. Our thanks are due to the 'Bayer', Leverkusen for a generous gift of Baytinal. The technical assistance of Mr. I. TANCINI in the experiments involving nerve section is gratefully acknowledged.

REFERENCES

ANSELL G. B. and RICHTER D. (1954) *Biochim. Biophys. Acta* **13,** 92.
CAVANAGH J. B., THOMPSON R. H. S. and WEBSTER G. R. (1954) *Quart. J. exp. Physiol.* **39,** 185.
JACOBS S. (1956) *The Analyst* **81,** 502.
JOHNSON A. C., MCNABB A. R. and ROSSITER R. J. (1949) *Biochem. J.* **45,** 500.
PARNAS K. J. and HELLER J. (1925) *Biochem. Zeitschr.* **155,** 247.
PORCELLATI G. and CURTI B. (1958) *IV International Congress of Biochemistry.*
PORCELLATI G. and THOMPSON R. H. S. (1957) *J. Neurochem.* **1,** 340.
WARBURG O. (1948) *Wasserstoffüberträgendefermente.* Werner Saenger Press, Berlin.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp 283 to 292. Pergamon Press Ltd. Printed in Northern Ireland

METABOLIC CONSEQUENCES OF LOCALIZED APPLICATION OF ELECTRICAL PULSES TO SECTIONS OF CEREBRAL WHITE MATTER

M. KUROKAWA*
Department of Biochemistry, Institute of Psychiatry
(British Postgraduate Medical Federation, University of London)
Maudsley Hospital, London, S.E.5

(*Received* 1 *October* 1959)

OL.
5
9/60

IT HAS been shown that sections of subcortical white matter as well as of cerebral grey matter are capable of responding to applied electrical pulses by changes in metabolism (BOLLARD and MCILWAIN, 1957). Although these parts of the brain differ greatly in structure, corresponding differences were not seen in metabolic response to distributed, maximally applied electrical stimuli. More recently, however, it has been observed that white matter is more sensitive than grey matter to locally applied electrical pulses (BOLLARD, to be published). Thus electrical stimuli which were applied to a limited area in white matter slices were found to evoke disproportionately large responses, which could be understood as a spread of the effect of the pulses to parts of slice distant from the electrodes.

In the present paper, evidence is given that the apparent spread of the effects of applied electrical pulses depended upon the direction of the nerve fibres in relation to the electrodes to which the pulses were applied. Increase in respiration caused by the spread of pulses proved to be more susceptible to changes in experimental conditions than that induced by maximal distributed electrical stimuli. Attention was also given to the effect on labile phosphate metabolism of locally and maximally applied electrical stimuli.

METHODS

Tissues and saline solutions. Tissues were taken from adult guinea pigs, stunned by a blow on the neck, and were prepared, with a blade and guide, as slices of defined thickness, 0·4 mm and 0·35 mm in white and in grey matter respectively. White matter slices were cut from the subcortical white matter after exposing the lateral ventricle and carefully removing the grey matter of the caudate nucleus along its striae. The remaining portion was then inverted so that the outer convexity of the cerebral cortex was uppermost, and slices of grey matter were cut from it. Unless otherwise stated, the first slice cut was used exclusively. Slices after cutting were floated in a shallow dish containing a saline solution, and trimmed to rectangles of about 50 mg in weight which fitted the grid electrodes, H and L used in vessel A (Fig. 1; AYRES and MCILWAIN, 1953). They were drained of saline, weighed on a torsion balance, fitted to their electrodes, and placed in the corresponding vessel containing 3·5 ml of the experimental saline. The length and shape of the slices are indicated in Fig. 2.

The phosphate–saline contained 126 mM-NaCl, 5·0 mM-KCl, 1·3 mM-KH_2PO_4, 1·3 mM-$MgSO_4$, 2·7 mM-$CaCl_2$ and 10 mM-sodium phosphate buffer. The substrate was 10 mM-glucose unless otherwise stated. The bicarbonate–saline contained 117 mM-NaCl, 4·7 mM-KCl, 1·2 mM-KH_2PO_4, 1·2 mM-$MgSO_4$, 2·6 mM-$CaCl_2$ and 25 mM-bicarbonate buffer. These were gassed with O_2 and with 95% N_2–5% CO_2 respectively. The glycylglycine–saline was the same as the phosphate–saline but with 30 mM-glycylglycine buffer replacing the phosphate buffer. All media were at pH 7·4.

* Present Address: Department of Neuropsychiatry, Faculty of Medicine, University of Tokyo, Tokyo.

Determinations and procedures. Oxygen uptake and carbon dioxide evolution were followed manometrically at 37·5°, with readings at 8 min and at 5 min intervals respectively. In anaerobic experiments, a piece of yellow phosphorus was placed in the centre well to ensure strictly anaerobic conditions. The manometers were placed in the thermostat 30–35 min after the death of the animal and equilibrated for 10 min. Electrical stimuli were usually applied between the 40th and 80th min of

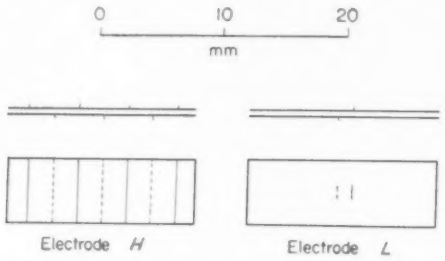


FIG. 1.—Schematic representation of the electrodes. H and L, showing their relationship to the tissue slices to which pulses are applied. Electrode H supplies distributed (D) stimuli, while electrode L localized (L) stimuli.

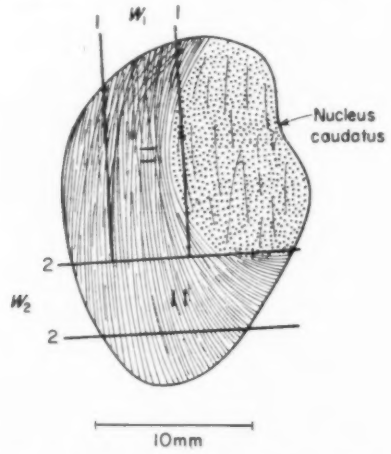


FIG. 2.—Schematic representation of a section from the cerebral white matter of the guinea pig (left hemisphere). The lines 1 and 2 show the direction along which slices were trimmed to produce the specimens placed in electrodes; = indicates the position of the L-type electrode in each trimmed slice.

incubation. They were supplied and measured by the apparatus of AYRES and MCILWAIN (1953); alternating condenser pulses of exponential voltage–time relationship, time constant 0·4 msec, and peak potential 12 v and 10 v for localized and distributed stimuli respectively, were applied at 100/sec.

In the experiments indicated, 1·75 M-KCl solution was added at the 40th min from a side-arm to give a 55 mM-solution; this caused an increase in osmotic pressure which was not corrected.

Lactic acid was determined according to the method of BARKER and SUMMERSON (1941), as modified by LEPAGE (1957). Generally 0·5 or 1·0 ml of the incubation medium was used for the determination.

Rapid changes in the creatine phosphate level in the slices on passage of electrical pulses were examined by means of the quick-transfer apparatus of HEALD and MCILWAIN (1956). The holder was slightly modified (MCILWAIN, 1960) by attaching fine nylon net to the plastic frame of the electrode jaws, just above the upper and below the lower silver wires, to keep the slices in position. Correct positioning of the electrode vis-à-vis the slice was particularly important for obtaining a good metabolic response when the localized electrode was used (Fig. 2). Slices of about 50 mg in weight were fitted to the holder electrodes and incubated in 5 ml glycylglycine–saline at 37·5° for 50 min prior to

VOL. 5 1959/6

stimulation. Immediately after stimulation for a definite period, slices were quickly transferred to 2·5 ml of ice-cold 10% (w/v) trichloroacetic acid. They were then drained of the acid for 1 sec, transferred to a small centrifuge tube of about 5 ml in volume, and quickly ground with a plunger in 2·5 ml ice-cold 10% (w/v) trichloroacetic acid. This double transfer was found to be necessary to avoid excessive contamination with inorganic phosphate from the medium adhering to the holder. Preliminary experiments showed that no appreciable loss of creatine phosphate occurred during the procedure if the tissues were ground within 5–6 sec after cessation of pulses. The suspension was then centrifuged at 2,500 r.p.m. for 7 min at −5°, and 2·0 ml of the supernatant fluid was used for phosphate analysis. Inorganic phosphate and phosphocreatine phosphate were determined in the barium-insoluble and in the barium-soluble, alcohol-insoluble fractions respectively, as described by HEALD (1954). No attempt was made to precipitate barium creatine phosphate before determination, since creatine phosphate was found to be the major labile phosphate present in extracts of cerebral cortex (MCILWAIN, BUCHEL and CHESHIRE, 1951) or of cerebral white matter (BOLLARD and MCILWAIN, 1957) which had respired in a glucose-containing saline solutions.

TABLE 1.—METABOLIC RESPONSE TO LOCALIZED ELECTRICAL STIMULI, TO DISTRIBUTED ELECTRICAL STIMULI, AND TO POTASSIUM CHLORIDE

Approximately 50 mg of tissue was incubated at 37·5° in 3·5 ml of phosphate-(aerobic) or bicarbonate-(anaerobic) saline at pH 7·4 with 10 mM-glucose as substrate. Localized (L) or distributed (D) condenser pulses (Peak potential: L, 12 V; D, 10 V; time constant, 0·4 msec; frequency, 100/sec, alternating) were applied during the 40th–80th min. KCl was added at 40th min, from side-arm, to give a 55 mM solution. Mean values are given ± standard errors; other values are the average of triplicates agreeing within 10 per cent.

Tissue	Applied agent	No.of expts.	Respiration*		Aerobic glycolysis†	No.of expts.	Anaerobic glycolysis‡	
			0–40 min	40–80 min			0–40 min	40–80 min
White matter	None	5	30 ± 3	29 ± 3	21 ± 1	3	105	87
	L	5	30 ± 3	48 ± 5	28 ± 1	3	101	80
	D	5	32 ± 2	57 ± 3	38 ± 2	3	100	53
	KCl	3	31	55	32	3	103	38
Grey matter	None	5	60 ± 3	60 ± 3	25 ± 2	3	155	140
	L	5	61 ± 5	67 ± 5	31 ± 2	3	151	129
	D	5	60 ± 3	112 ± 4	61 ± 4	3	152	74
	KCl	3	62	118	52	3	155	25

* μmoles O_2/g of fresh tissue per hr.

† μmoles lactic acid/g of fresh tissue per expt (80 min), determined chemically after the respiratory measurements of the preceding columns.

‡ μmoles CO_2/g of fresh tissue per hr, determined manometrically.

RESULTS

Comparison of metabolic response. Aerobic lactic acid formation in slices from cerebral white matter is, unlike respiration, comparable in magnitude to that in grey. The glycolytic response to maximally applied electrical stimuli or to the addition of the KCl was less in white matter than in grey. The rate of anaerobic glycolysis in white matter slices was found to be about two-thirds that in grey (outer); each fell approximately 10–15 per cent after 40 min and was diminished to the same extent by applied pulses (Table 1).

Electrical stimuli which were applied to a limited area of white matter slice (Fig. 2) caused a disproportionately large increase in respiration which was more in accord with a spread of pulses to 60–70 per cent of the slice (Table 1; BOLLARD, to be published). The same trend was also seen in aerobic lactic acid formation, but this was less definite under the conditions used in the present experiment. In the slices of grey matter, the percentage increase in respiration using localized stimuli was more in accord with the area immediately adjacent to the electrodes. In both tissues, anaerobic

TABLE 2.—RESPIRATORY RESPONSE OF SLICES TRIMMED IN DIFFERENT DIRECTIONS

Experimental conditions were the same as in Table 1. W_1 and W_2, both from white matter, were cut as shown in Fig. 2. I was the first (outer), II was the second slice from grey matter. In I (1), the long side of slice trimmed to rectangle was parallel with the interhemispherical fissure, while in I (2) it was transverse. Mean values are given ± standard error; other values are the average of triplicates which agreed within 7 per cent.

Tissue	Slice	Pulses	No. of expts.	Respiration (μmoles/g per hr) 0–40 min	40–80 min	Dif.
White matter	W_1	L	4	30 ± 1	51 ± 1	21*
	W_2	L	4	29 ± 2	40 ± 5	11*
	W_1	D	4	30 ± 2	55 ± 3	25
	W_2	D	4	32 ± 2	57 ± 3	25
Grey matter	I (1)	L	3	60	67	7
	I (2)	L	3	61	68	7
	II	L	3	58	66	8

* $P < 0.02$ (Student's 't' test) for the difference between W_1 and W_2 with pulses type L.

glycolysis was diminished by about 15–20 per cent during the localized stimulation, a change which could hardly be differentiated from the spontaneous decrease in rate without stimuli.

Structure of slices. The sheet of white matter available (Fig. 2) may be trimmed in different ways to give specimens which differ in structure. Although the rate of respiration prior to stimulation was the same in slices differently trimmed, increase in respiration with locally applied pulses differed significantly in slices that were differently trimmed (Table 2). This was not the case with comparable slices obtained from grey matter. It seemed that the effect on respiration caused by the apparent spread of pulses might reflect the structural peculiarities of the white matter.

The direction of the nerve fibres in white matter slices has been examined in frozen sections of 8–10 μ in thickness, stained with haematoxylin (Fig. 2). In slice W_1, which showed the highest respiratory response, nerve fibres were found to lie nearly parallel to the long sides of the trimmed slices. In slice W_2 they ran perpendicularly to the length of the slice. It appears, therefore, that the apparent spread of the applied pulses is related to the direction of the nerve fibres in the slice.

VOL. 5 1959/6

Differences in magnitude of the respiratory response according to the positioning of the electrode, were not seen in white matter slices stimulated with distributed pulses (Table 2).

Substrate concentration. Lowering the glucose concentration has a differential effect on the respiratory and glycolytic responses to applied pulses, as reported by McIlwain (1953*a*). In the present experiment, it was again confirmed that lowering

Table 3.—Changes in metabolic response with lowered glucose level
Experimental conditions as in Table 1. Values are means, some followed by standard error; others are the average of duplicates agreeing within 7 per cent.

Tissue	Pulses	Glucose level (mM)	No. of expts.	Respiration (μmoles/g per hr)			Aerobic glycolysis (μmoles/g per 80 min)
				0–40 min	40–80 min	Dif.	
White matter	L	10	5	30 ± 3	50 ± 3	20*	27 ± 2**
	L	4	2	34	52	18	25
	L	2	5	34 ± 2	40 ± 1	6*	17 ± 1**
	L	0	2	26	26	0	
	D	10	4	32 ± 2	57 ± 3	25	39 ± 2†
	D	4	2	33	52	19	33
	D	2	4	30 ± 3	53 ± 4	23	25 ± 1†
	D	0	2	26	25	−1	
Grey matter	D	10	2	60	108	48	65‡
	D	4	2	60	112	52	54
	D	2	2	58	109	51	33‡
	D	0	2	41	36	−5	

* And ** $P < 0{\cdot}001$.
† And ‡ $P < 0{\cdot}02$.

the glucose level from 10 to 2 mM reduced the glycolytic response but was without effect on the respiratory response (Table 3). This was observed both in white matter and in grey, when maximally stimulated.

The respiratory response caused by the spread of applied pulses, however, proved to be more susceptible to changes in glucose level than that induced by maximally applied pulses. Thus, lowering the glucose level from 10 to 2 mM reduced the respiratory response by nearly 70 per cent with localized stimulation but was without effect on the response to maximally applied pulses (Table 3).

Creatine phosphate level. Creatine phosphate was found to be resynthesized in slices fitted to the modified holder to the same extent as in previous observations with slices in the original holder. With glucose as substrate, the level of creatine phosphate attained in white matter (0·98 μmoles/g) was about 70 per cent of that in the cerebral cortex (Table 4). This was in good agreement with the results obtained in manometric vessels (Bollard and McIlwain, 1957).

The rates of net loss of phosphocreatine with distributed pulses were found to be about 550 and 900 μmoles/g per hr in white and in grey matter respectively. No changes were observed in creatine phosphate level following the localized stimulation of slices. Further, no changes occurred even with a higher peak potential of 18 v.

TABLE 4.—CHANGES IN CREATINE PHOSPHATE LEVEL ON PASSAGE OF ELECTRICAL IMPULSES

Slices were kept in modified quick-transfer holders and incubated in 5 ml of saline at 37·5° for 50 min prior to stimulation. Saline was buffered with 30 mM-glycylglycine at pH 7·4 and contained 1·3 mM KH_2PO_4. Means were from two or three experiments which agreed within 9 per cent. For details see Methods and for abbreviations see Table 1.

Tissue	Stimulation Type	Stimulation Period (sec)	No. of expts.	Creatine phosphate (μmoles P/g)	Inorganic phosphate (μmoles P/g)
White matter	(Before incubation)		3	0·34	5·90
	(After incubation)		6	0·98 ± 0·03	3·76 ± 0·18
	L, 12 v	5	4	1·06 ± 0·12	3·53 ± 0·15
	L, 12 v	60	3	1·01	3·52
	L, 18 v	60	2	0·96	3·59
	D, 10 v	2	3	0·98	3·83
	D, 10 v	5	3	0·63	4·13
	D, 10 v	60	2	0·58	4·30
Grey matter	(Before incubation)		3	0·43	6·66
	(After incubation)		6	1·36 ± 0·08	2·95 ± 0·17
	L, 12 v	5	3	1·39	3·33
	L, 12 v	60	3	1·31	2·94
	L, 18 v	60	2	1·33	2·90
	D, 10 v	2	3	1·32	2·84
	D, 10 v	5	3	0·71	3·72
	D, 10 v	60	2	0·69	3·88

L-Glutamic, γ-aminobutyric, DL-γ-amino-β-hydroxybutryic acids and the respiratory response. Unlike glutamic acid, γ-aminobutyric or γ-amino-β-hydroxybutyric acid did not, by themselves, increase oxygen uptake by cerebral tissues of the guinea pig. However, respiration with 10 mM-glucose as substrate could be slightly enhanced (by about 5–10 μmoles/g per hr) in both white and grey matter tissues by the addition of 10–20 mM-γ-aminobutyric or γ-amino-β-hydroxybutyric acids (Table 5).

A differential effect on the stimulated respiration was seen with the two butyric acid derivatives. The respiratory response in glucose-containing media was not influenced by the addition of 10 mM-γ-aminobutyric acid, irrespective of the nature of the pulses. On the other hand, 10 mM-γ-amino-β-hydroxybutyric acid incorporated in the glucose-containing media reduced the respiratory response when it was induced

VOL. 5 1959/6

by localized stimuli; this inhibition was observed even with a concentration of 5 mM. However, differences which depended upon the nature of pulses could not be observed when a higher concentration of the acid was used; thus the respiratory responses to distributed as well as to localized stimuli were similarly inhibited when 20 mM-γ-amino-β-hydroxybutyric acid was added to the medium.

TABLE 5.—L-GLUTAMIC, γ-AMINOBUTYRIC, DL-γ-AMINO-β-HYDROXYBUTYRIC ACIDS AND RESPIRATORY RESPONSE

In systems marked with asterisk, 10 mM-glucose was omitted from the basic phosphate media. Means were from duplicates or triplicates agreeing within 10 per cent. Other details as in Table 1.

Tissue	Pulses	Addition	(mM)	No. of expts.	Respiration (μmoles/g per hr)		
					0–40 min	40–80 min	Dif.
White matter	L	None*		2	26	25	−1
	L	None		6	29 ± 3	49 ± 5	20
	L	Glutamic*	20	2	43	45	2
	L	Glutamic	20	2	40	39	−1
	L	Aminobutyric*	10	3	25	23	−2
	L	Aminobutyric	10	3	38	60	22
	L	Aminohydroxybutyric*	20	3	24	24	0
	L	Aminohydroxybutyric	20	3	35	32	−3
	L	Aminohydroxybutyric	10	3	34	39	5
	L	Aminohydroxybutyric	5	4	35 ± 1	42 ± 2	7
	D	None		6	29 ± 2	54 ± 3	25
	D	Aminohydroxybutyric	10	3	36	49	13
Grey matter	D	None*		2	44	42	−2
	D	None		6	60 ± 2	112 ± 6	52
	D	Glutamic*	20	2	75	64	−11
	D	Glutamic	20	2	76	73	−3
	D	Aminobutyric*	10	3	48	45	−3
	D	Aminobutyric	10	3	62	115	53
	D	Aminohydroxybutyric*	10	3	46	46	0
	D	Aminohydroxybutyric	20	3	72	90	18
	D	Aminohydroxybutyric	10	3	62	106	44

Changes in metabolic response by addition of eserine and acetylcholine. Eserine at 3×10^{-5}M, which inhibits choline esterase activity, reduced the respiratory response to localized stimuli in white matter tissues by about 75 per cent without appreciably affecting aerobic lactic acid formation (Table 6). Inhibition was also observed with the combined use of 3×10^{-5} M-eserine and 5×10^{-5} M-acetylcholine, but no

additional effect was produced by the acetylcholine. The response induced in white matter by maximally applied pulses was not influenced at all even with a higher concentration of eserine (10^{-4} M). Similarly, no inhibition with 10^{-4} M-eserine was seen in grey matter slices stimulated with distributed pulses; this was in agreement with results previously reported (McILWAIN, 1951).

TABLE 6.—METABOLIC RESPONSE IN PRESENCE OF ESERINE, ACETYLCHOLINE AND OF A GUANIDINE COMPOUND

Eserine sulphate, acetylcholine bromide and 1:2-di-α-naphthyl-3-(3-oxapentamethylene)-guanidine hydrochloride were present in the salines indicated, from the beginning of experiment. Means are from three experiments which agreed within 8 per cent. Other details as in Table 1.

Tissue	Pulses	Addition (M)	No. of expts.	Respiration (μmoles/g per hr)			Aerobic glycolysis (μmoles/g per 80 min)
				0–40 min	40–80 min	Dif.	
White matter	L	None	6	29 ± 3	45 ± 4	16*†‡	26 ± 4
	L	Eserine 3×10^{-5}	6	28 ± 2	32 ± 3	4*	26 ± 3
	L	Eserine 3×10^{-5} ACh 5×10^{-5}	4	29 ± 3	38 ± 3	9†	25 ± 2
	L	Guanidine 5×10^{-7} derivative	3	33	39	6‡	25
	D	None	4	34 ± 2	58 ± 2	24	39 ± 1
	D	Eserine 10^{-4}	4	31 ± 1	53 ± 4	22	36 ± 2
	D	Guanidine 5×10^{-7} derivative	3	32	57	25	36
Grey matter	D	None	4	60 ± 3	112 ± 4	52	61 ± 2
	D	Eserine 10^{-4}	4	58 ± 2	119 ± 3	61	64 ± 2
	D	Guanidine 10^{-5} derivative	3	48	62	(14)	39
	D	Guanidine 5×10^{-6} derivative	3	50	85	(35)	45
	D	Guanidine 5×10^{-7} derivative	3	57	109	52	61

* $P < 0.001$.
† $P < 0.01$.
‡ $P < 0.05$.

A guanidine compound and metabolic response. 1:2-Di-α-naphthyl-3-(3-oxapentamethylene)-guanidine hydrochloride, a synthetic local anaesthetic which proved to be as potent as nupercaine when examined by the rabbit cornea test (BELL, P. H., personal communication), was found to be a very potent inhibitor of the respiration of cerebral tissues. When the compound was present in the test media at a concentration of 10^{-5} M, respiration of grey matter prior to stimulation was inhibited by about 20 per cent, while the respiratory response was completely suppressed. The glycolytic response was also inhibited, but to a lesser extent. At 5×10^{-7} M, the metabolic response to maximally applied pulses was no longer influenced, but the respiratory response to localized stimuli was still inhibited by about 60 per cent (Table 6).

DISCUSSION

The fact that the respiration of white but not of grey matter slices markedly increases in rate in response to locally applied electrical pulses strongly suggests that structural aspects of the tissues are involved. It has been found that increase in

respiration with localized stimulation differs significantly according to the positioning of electrodes, and that, of two positions, the higher rate is obtained with the electrodes in a position in which they are likely to affect a greater length of nerve fibres in the slice. Such differences were not seen when stimuli were distributed over the whole of the slice: it is understandable that a specific effect caused by the spread of pulses along nerve fibres would not be seen when the whole of the slice was exposed to a voltage gradient sufficient for maximal stimulation.

The effect of the spread of pulses can be demonstrated with glucose but not with glutamic acid as substrate, in aerobic but not in anaerobic conditions. It is more sensitively conditioned by availability of substrate than is the response to maximal stimuli. Lowering the glucose level from 10 to 2 mM reduced the respiratory response by nearly 70 per cent with localized stimulation but not with a distributed one.

It has been confirmed that the stimulated metabolism of separated cerebral tissues is more sensitive to added agents than is their metabolism in ordinary conditions (McIlwain, 1953*b*). Respiration of white matter subjected to localized stimulation proved to be even more sensitive to some substances. Thus, 3×10^{-5} M-eserine and 5×10^{-7} M-1:2-di-α-naphthyl-3-(3-oxapentamethylene) guanidine, when incorporated in the test media, affected the respiratory responses of white matter to localized but not to distributed stimuli. It is clearly indicated that the differences in sensitivity to added agents is not due to the difference in tissues, since white matter and grey matter behave similarly towards the agents when stimulated with maximal pulses. However, much remains to be studied in order to specify whether the differential sensitivity is exclusively connected with the manner of stimulation, or whether it also depends to some extent on the different pharmacological properties of the agents examined.

In the present experiments, the rate of phosphocreatine breakdown in grey matter tissues subjected to maximal stimulation was found to be approximately 900 μmoles/g per hr. This was about 30 per cent lower than the maximum rate of 1,200–1,400 μmoles/g per hr found by Heald (1954), which might be attributed to the lowered peak potential applied in the present experiment. The rate of phosphocreatine breakdown in white matter on passage of maximal stimuli was approximately 60 per cent of that in grey matter, which was comparable with the ratio of the concomitant increase in respiration in the two tissues (Table 1). If the apparent spread of pulses in white matter accompanies the release of labile phosphate in the same manner as it does with maximally applied pulses, approximately 0·2–0·25 μmoles/g of creatine phosphate would have been expected to disappear while applying pulses, but this was not detected in the present experiment. The failure to demonstrate changes in creatine phosphate level might suggest a different balance between the release and the recovery processes of the labile phosphate under the two different potential conditions. To elucidate such differences further study is necessary.

The difference in effect on the stimulated metabolism of the two butyric acid derivatives, γ-aminobutyric and γ-amino-β-hydroxybutyric acids, is interesting. Attention is drawn to the recent study by Hayashi (1959), where some evidence was given that the inhibitory factor was L-γ-amino-β-hydroxybutyric acid rather than γ-aminobutyric acid. Although such a finding is essentially in agreement with the present work, no attempt at assessment is made because of major differences between the two experimental systems. The matter is complicated by the metabolism of the

acids by the tissues of guinea pig in glucose-containing media (Table 5; TSUKADA, NAGATA and TAKAGAKI, 1957).

SUMMARY

(1) Changes in respiration, glycolysis, and in the levels of inorganic phosphate and phosphocreatine induced by electrical pulses, have been examined in slices from the cerebral white matter of guinea pig, with special reference to the local application of pulses.

(2) A disproportionately large increase (55–65 per cent) in the respiration of white matter in response to locally applied electrical pulses was considered to be due to an apparent spread of pulses in the tissue. It has been demonstrated that the spread is related to the direction in which the nerve fibres were found to lie in the slices.

(3) The metabolic effect associated with the spread of applied pulses proved to be more susceptible to added agents, such as 3×10^{5} M-eserine, 5×10^{-3} M-γ-amino-β-hydroxybutyric acid and 5×10^{-7} M-1:2-di-α-naphthyl-3-(3-oxapentamethylene)-guanidine, than that induced by electrical pulses distributed so as to give maximal metabolic responses.

(4) Similarly, metabolic sequelae to locally applied pulses were more sensitive to the lowering of the glucose concentration from 10 to 2 mM. Phenomena attributable to spread of applied pulses were not seen in anaerobic conditions.

(5) No change was detected in the creatine phosphate level with localized stimuli applied to slices of white or of grey matter. The rate of phosphocreatine breakdown in white matter induced by maximally applied pulses was found to be about 60 per cent of that in grey matter.

Acknowledgements—The author is greatly indebted to Professor H. MCILWAIN for his generous support and criticism during the work and for help in preparing the manuscript. He also wishes to thank Dr. J. B. BRIERLEY for the histological examination of certain tissues, Miss B. M. BOLLARD for technical advice, and Dr. P. H. BELL, Biochemical Research Section, Lederle Laboratories, U.S.A., for a generous supply of 1:2-di-α-naphthyl-3-(3-oxapentamethylene)guanidine hydrochloride.

The work was carried out during the tenure of a British Council Scholarship.

REFERENCES

AYRES P. J. W. and MCILWAIN H. (1953) *Biochem. J.* **55,** 607.
BARKER S. B. and SUMMERSON W. H. (1941) *J. biol. Chem.* **138,** 535.
BOLLARD B. M. To be published.
BOLLARD B. M. and MCILWAIN H. (1957) *Biochem. J.* **66,** 651.
HAYASHI T. (1959) *J. Physiol.* **145,** 570.
HEALD P. J. (1954) *Biochem. J.* **57,** 673.
HEALD P. J. and MCILWAIN H. (1956) *Biochem. J.* **63,** 231.
LEPAGE G. A. (1957) *Manometric Techniques* (Edited by UMBREIT W. W., BURRIS R. H. and STAUFFER J. F.) p. 275, Burgess, Minneapolis.
MCILWAIN H. (1951) *Brit. J. Pharmacol.* **6,** 531.
MCILWAIN H. (1953*a*) *Biochem. J.* **55,** 618.
MCILWAIN H. (1953*b*) *Biochem. J.* **53,** 403.
MCILWAIN H. (1960). In preparation.
MCILWAIN H., BUCHEL L. and CHESHIRE J. D. (1951) *Biochem. J.* **48,** 12.
TSUKADA Y., NAGATA Y. and TAKAGAKI G. (1957) *Proc. imp. Acad. Japan* **33,** 510.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 293 to 296. Pergamon Press Ltd. Printed in Northern Ireland

THE FORM IN WHICH CHOLESTEROL OCCURS IN THE ADULT CNS

C. W. M. ADAMS and A. N. DAVISON
Department of Pathology, Guy's Hospital Medical School, London, S.E.1.

(*Received* 7 *October* 1959)

IN A previous paper (ADAMS and DAVISON, 1959) it was shown, by chemical analysis and histochemistry, that there is a time, early in the development of the myelin sheath, when a large part of myelin cholesterol is present in ester form. These high ratios of ester to free cholesterol during myelination led us to consider whether the ester may play an important, if unknown, part in myelinogenesis. This role could be that of a precursor for the formation of free cholesterol in the sheath. Alternatively the esters may be incorporated into myelin at an early stage of myelination and remain there intact. If the former hypothesis is correct cholesterol esters should disappear from the adult myelin sheath while, if the latter be true, ester should be recovered from adult white matter. In the second case the ester would, however, be considerably diluted by the accumulation of large amounts of 'adult' myelin lipids now present.

In this paper combined chemical and chromatographic analyses for free and esterified cholesterol have been made on adult human brain and cord. It will be shown that there is no esterified cholesterol in the adult CNS and that these esters cannot be detected by histochemical means in the mature myelin sheath.

METHODS AND RESULTS

Normal human cerebral white matter (corpus callosum) and cord were obtained at post-mortem. The tissues were extracted in twenty times their volume of chloroform–methanol (2 : 1 v/v). After filtering and washing with water (FOLCH, LEES and SLOANE-STANLEY, 1957) suitable portions of the chloroform phase were removed for chromatographic separation and analysis for free and esterified cholesterol (see ADAMS and DAVISON, 1959). In one series of experiments analyses were made on the crude filtered extract before and after disruption of proteolipids (FOLCH and LEES, 1951). After washing, analyses were then made on the upper (aqueous) phase, interfacial fluff (proteolipid) and lower (chloroform) phase. Further analyses were made on the interfacial fluff and chloroform phase after chromatographic separation.

The results are presented in Tables 1 and 2. In one sample of brain a trace of cholesterol ester was detected, but this was only 1·4 per cent of the total cholesterol. In the six other brains and in the six cords no esterified cholesterol was found. In the earlier paper (ADAMS and DAVISON, 1959) cholesterol esters were shown to be eluted from the alumina column by the first solvent mixture. In this present work apparent traces of ester eluted by the final solvent mixture have been regarded as due to errors of estimation and, accordingly, ignored.

Histochemistry. To be certain that none of the tissues analysed were undergoing demyelination, representative samples of each were fixed in 4 per cent formaldehyde–saline, cut on the freezing microtome and stained by the OTAN method (modified

TABLE 1.—CHROMATOGRAPHIC SEPARATION OF FREE AND ESTERIFIED CHOLESTEROL IN HUMAN ADULT BRAIN (CORPUS CALLOSUM) AND CORD

Chloroform–methanol (2:1 v/v) extracts of CNS were washed with water and, after concentration, separated by column chromatography before analysis. Solvents A, B and C were 1%, 3% and 30% (v/v) ether in petroleum spirit (b.p. 40–60°) respectively. Cholesterol esters are eluted in fraction A. The apparent traces of ester in fraction C may result from errors in the method of analysis by difference.

Age of subject (years)	Fraction from column	Corpus callosum		Cord	
		cholesterol ester (mg/g wet wt)	free cholesterol (mg/g wet wt)	cholesterol ester (mg/g wet wt)	free cholesterol (mg/g wet wt)
13	A	0*	0	0	0
	B	0	0	0	0
	C	0·2	4·9	0·9	5·00
31	A	0	0	0	0
	B	0	0	0	0
	C	0·3	5·4	0·2	5·4
52	A	0	0	0	0
	B	0	0	0	0
	C	0	7·1	0	11·2
57	A	0	0·2		
	B	0	0	—	—
	C	0	3·5		
59	A	0	0	0	0
	B	0	0	0	0
	C	0·05	3·9	0·7	4·9
66	A	0·1	0·2	0	0
	B	0	0	0	7·6
	C	0·05	7·0	0	5·6
69	A	0	0	0	0
	B	0	0	0	0
	C	0·05	5·6	0	4·8
79	A	0	0	0	0
	B	0	0	0	0
	C	1·2	5·9	5	4·6

* A value of 0 indicates less than 0·01 mg/g wet wt.

Marchi reaction: ADAMS, 1959). All the tissues analysed were shown to be normal: no Marchi-positive material (esterified cholesterol) was seen in them.

DISCUSSION

Esterified cholesterol has previously been found in the developing CNS of man and chick (ADAMS and DAVISON, 1959). Histochemical evidence suggested that a large

VOL. 5 1959/6

part of the ester was in the newly-formed myelin sheath. In this previous study esterified cholesterol was first separated chromatographically so that its subsequent analysis became correspondingly more sensitive. SPERRY (1957) observes that "the presence of cholesterol esters in appreciable quantities in the developing brain would be of much interest, but (hitherto) the small number, high variability, and random

TABLE 2.—EFFECT OF PRELIMINARY TREATMENT ON CHOLESTEROL ANALYSES OF CORPUS CALLOSUM EXTRACTS

The corpus callosum of a 54 year old man was extracted with chloroform–methanol (2:1 v/v). Washing and chromatographic procedures were as described in the text.

Extract or fraction		Cholesterol ester (mg/g wet wt)	Free cholesterol (mg/g wet wt)
1. Crude chloroform–methanol extract		0	6·15
2. Crude extract heated with 6% water to decompose proteolipid		0·45	6·5
After equilibration with water			
3. Upper phase (aqueous)		0	0
4. Interfacial fluff (proteolipid)		0·09	1·0
5. Lower phase (chloroform)		0	6·15
6. Column chromatography of 4.	A	0	0
	B	0	0
	C	0	0
7. Column chromatography of 5.	A	0	0
	B	0	0
	C	0	6·35

nature of the data do not permit such a conclusion". He goes on to say that "it is generally believed that little, if any, cholesterol, combined as ester, is present in the adult brain, but since combined cholesterol is usually determined as the difference between total and free cholesterol, establishing the complete absence of cholesterol esters is difficult". (Data on the amount of esterified cholesterol in the developing and adult CNS have been presented by JOHNSON, MCNABB and ROSSITER, 1948; BRANTE, 1949; and CUMINGS, 1953, 1957).

For these reasons it seemed appropriate to apply our more sensitive method of analysis to the problem whether or not esterified cholesterol is present, even in traces, in the adult CNS. Apart from a trace of ester in one adult human brain, the other 12 samples of CNS contained no esterified cholesterol. This can only mean that the ester laid down in developing myelin is subsequently removed or de-esterified. It is suggested that esterified cholesterol may be a substrate for the free cholesterol of myelin. This interpretation would accord with the views of UZMAN and RUMLEY (1958) who claim to have shown that the lipids laid down in developing myelin were already present, as such or as precursors, in unmyelinated white matter.

SUMMARY

1. The amount of esterified cholesterol in the adult human CNS has been determined by combined chomatographic separation and chemical analysis.

2. A trace of esterified cholesterol was found in one brain (corpus callosum) but 6 other brains and 6 cords contained none.

3. Since cholesterol ester is present in developing myelin, its absence from mature myelin suggests that it plays a metabolic role in myelinogenesis.

Acknowledgements—The authors wish to acknowledge the interest shown in this work by Professor G. PAYLING WRIGHT and the technical assistance of Miss OLGA BAYLISS.

REFERENCES

ADAMS C. W. M. (1959) *J. Path. Bact.* **77,** 648.
ADAMS C. W. M. and DAVISON A. N. (1959) *J. Neurochem.* **4,** 282.
BRANTE G. (1949) *Acta physiol. scand.* **18,** Suppl. 63.
CUMINGS J. N. (1953) *Brain* **76,** 551.
CUMINGS J. N. (1957) In *Cerebral Lipidoses* (Edited by CUMINGS J. N.) p. 112. Blackwell, Oxford.
FOLCH J. and LEES M. (1951) *J. biol. Chem.* **191,** 807.
FOLCH J., LEES M. and SLOANE-STANLEY G. H. (1957) *J. biol. Chem.* **226,** 497.
JOHNSON A. C., MCNABB A. R. and ROSSITER R. J. (1948) *Biochem. J.* **43,** 573.
SPERRY W. M. (1957) in *Neurochemistry* (Edited by ELLIOTT K. A. C., PAGE I. H. and QUASTEL J. H.) p. 239. Thomas, Springfield, Illinois.
UZMAN L. L. and RUMLEY M. K. (1958) *J. Neurochem.* **3,** 170.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 297 to 303. Pergamon Press Ltd. Printed in Northern Ireland

VASOPRESSIN BIOSYNTHESIS—I. *IN VIVO* STUDIES*

HOWARD SACHS
Western Reserve University School of Medicine,
Department of Physiology

(*Received* 5 *October* 1959)

CERTAIN aspects of the physiological role and importance of the neurons connecting the hypothalamus and the neurohypophysis have been well established. (For a recent symposium and a review see HELLER, 1957, and SLOPER, 1958). In addition, it would now appear that neurosecretory cells may function as the final common pathway for the transmission of messages to certain organs of internal secretion (cf. BARGMANN *et al.*, 1958). However, practically nothing is known about the cellular events involved in these processes. It was, therefore, considered desirable to undertake studies designed to cast some light on the biochemical mechanisms involved in the biosynthesis, storage, and release of the neurohumor, vasopressin. A preliminary report has already been published (SACHS, 1959). Apart from the neurochemical and endocrinological considerations, the importance of such studies derives from the knowledge of the structure (cf. DU VIGNEAUD, 1955) and bond types inherent in the vasopressin molecule and thereby offers the possibility of contributing to the general problem of protein synthesis.

In this paper some *in vivo* studies in the dog are reported, concerned with the formation and isolation of ^{35}S-labelled vasopressin.

MATERIALS AND METHODS

Purification of vasopressin from the hypothalamus and neurohypophysis of the dog

Extraction from tissue. Six male dogs, each weighing between 12 and 15 kg, were sacrificed by a rapid intravenous injection of a 50% $MgSO_4$ solution (VAN DYKE *et al.*, 1955). The combined hypothalamic tissues were placed in 25 ml of ice cold 0·1 M-acetic acid, minced with scissors, and homogenized in an all-glass Potter–Elvehjem homogenizer (Step 1). The homogenate was immersed in a boiling water bath for 5 min, cooled, and centrifuged at 2000 **g**. The precipitate was resuspended in 15 ml of 0·1 M-acetic acid maintained at 100° for 3 min, cooled, and centrifuged. The precipitate was washed once more with 10 ml of acetic acid. The supernatant solutions were combined (Step 2) and enough 50% trichloroacetic acid (TCA) was added to bring the final concentration of TCA to 10%. The protein precipitating out of solution was centrifuged and washed once with 10% TCA and twice with 5% TCA. These TCA supernatant solutions were combined and treated with 3 equal volumes of ether to give a clear aqueous solution (Step 3) ready for ion exchange chromatography.

The posterior pituitaries obtained from these animals were also combined and treated in a manner similar to that described above for the hypothalamic tissue (with, however, one half the volume of acetic acid).

At each stage of purification (except No. 1) portions were removed for bioassay and protein determinations. Assays for pressor activity were carried out by means of the rat blood pressure method (Pharmacopeia of U.S. 1955) and the USP posterior pituitary Reference Standard which had a stated potency of 0·47 U/mg. Protein was determined (LOWRY *et al.*, 1951), with crystalline bovine serum albumin as a standard. The precipitated proteins (Steps 2 and 3) were dissolved in M-NaOH and estimated as described above.

Ion exchange chromatography. Carboxymethyl cellulose was prepared (ELLIS and SIMPSON, 1956)

* Supported in part by a Grant (No. A2650) from the National Institutes of Health, United States Public Health Service, and from the Foundation for the Study of Diabetes and Related Metabolic Disorders.

and had a capacity of 0·5 m-equiv/g. The aqueous extracts from Step 3 were adjusted to pH 6·0 (glass electrode) and chromatographed on columns 0·9 × 40 cm by means of a continuous gradient elution procedure (WARD and GUILLEMIN, 1957). The elution gradient was from 0·02 M-(pH 6·0) to 0·2 M-(pH 7·0) ammonium acetate. The biologically active peak (Step 4) was assayed for total pressor Units, and after removal of ammonium acetate by lyophilization, separate portions were used for the determination of protein content and subsequent chromatography on an XE-64 ion exchange resin. The lyophilized material was taken up in a little water, adjusted to pH 5·0, and placed on a 0·9 × 20 cm column of XE-64 resin (prepared according to HIRS *et al.*, 1953), and the elution of vasopressin was carried out as previously described (ACHER *et al.*, 1958). The gradient in this case was from 0·05 M-(pH 5·0) to 0·5 M-ammonium acetate (pH 7·7). The active peak (Step 5) was lyophilized repeatedly; the residue was taken up in 3–4 ml of water, adjusted to pH 5·0, and passed through a 0·9 × 10 cm column of carboxymethyl cellulose in the hydrogen form. Ammonium acetate (0·02 M, pH 6·0) was passed through the column at a rate of 10 ml/hr until the effluent pH was the same as the influent. A gradient of 0·2 M-buffer (pH 7·0) was started and vasopressin was eluted (Step 6) as previously described (WARD and GUILLEMIN, 1957). For Steps 5 and 6, blank columns were run and the Folin colour was determined for the effluents corresponding in position and volume to the biologically active peaks.

Incorporation experiments

Preparation of ^{35}S*-labelled cystine.* This was prepared by a modification of the procedure described by COWIE *et al.* (1952), *E. coli*, strain B (generously supplied by Dr. JOHN SPIZIZON) was grown in the presence of [^{35}S] sulphate obtained on allocation from the Atomic Energy Commission. The incubation medium was the same as the medium S of COWIE *et al.* (1952) and contained in addition, 0·3 μg of unlabelled S (as sodium sulphate), 32 μg of methionine sulphur, and 0·06–0·08 mc of carrier free [^{35}S] sulphate, per ml of medium. After 20 hr of growth at 37° in a rotary shaker, the cells were sedimented at 10,000 g (Sorvall refrigerated centrifuge) for 10 min. The supernatant solution was used for a second growth after the further addition of glucose and unlabelled methionine. The combined harvested cells were treated with TCA, and the bacterial protein was washed as described previously for ^{14}C-labelled liver proteins (SACHS, 1957). The air-dried protein (150–200 mg). was suspended in 50 ml of redistilled HCl (approximately 6 N) and treated for 20 hr at 110° under nitrogen. The HCl was removed *in vacuo* with a rotary evaporator (with repeated additions of water), and the residue taken up in a small volume of water. Portions were taken for determination of radioactivity, chromatography on paper (ROBERTS *et al.*, 1957) and on Dowex 50 (HIRS *et al.*, 1954), and the remainder of the hydrolysate was used for incorporation experiments in the dog. Radioactive materials were plated at infinite thinness on stainless steel planchets, and counting was performed with a thin window Geiger–Muller tube and scaler (Nuclear Instrument and Chemical Corporation).

Formation and isolation of ^{35}S*-labelled vasopressin.* Mongrel dogs (5–6 kg) were anaesthetized by the intravenous administration of 3–5 ml of pentothal sodium (2·5% solution) and a polyethylene cannula was inserted into the right jugular vein. The animal was replaced in its cage with the cannula leading through a heavy collar out behind the back of its neck through a hollow metal cable leading out of the cage and attached to a motor driven syringe calibrated to deliver 4 ml/hr. Heparinized saline was infused for 3 hr after the time that the animal awakened (which usually took about 1 hr), ^{35}S-labelled cystine (contained in the protein hydrolysate, 0·5–1·2 × 10^{10} counts/min) in heparinized saline was infused at the same rate for the desired length of time. After the infusion was complete, a 50% $MgSO_4$ solution was injected into the dog via the jugular vein cannula and the hypothalamus and neurohypophysis were excised and prepared separately for the isolation of labelled vasopressin. The purification procedure was identical to the one described above except that approximately 1 mg of highly purified beef (arginine) vasopressin was added as carrier to each tissue extract (TCA supernatant fluid prior to ether extraction). Carrier vasopressin was prepared by chromatography of partially purified beef vasopressin (65 Units/mg, kindly supplied by Wilson and Co.) on a 0·9 × 30 cm column of carboxymethyl cellulose as described above. The estimated potency of this material was 300–400 Units/mg. Paper ionophoresis of labelled vasopressin obtained at Step 5 was performed essentially as described by ACHER *et al.* (1952) at 4° with 0·05 M-pyridine acetate at pH 6·5. Performic acid oxidation of this material (obtained at Step 5) was carried out (DU VIGNEAUD *et al.*, 1953) and paper ionophoresis of the oxidized material was performed as described above.

VOL.
5
1959/6

RESULTS

Preparation of ^{35}*S-labelled cystine.* Under the selected conditions of growth radio-sulphate was incorporated into organic, protein-bound sulphur with a total yield of 60–80 per cent. After acid hydrolysis of this ^{35}S-labelled protein followed by chromatography on Dowex 50 (HIRS *et al.*, 1954) 60–70 per cent of the incorporated counts could be recovered in the cystine (*meso* and DL) peak. This material had a specific activity* of 7·0 to 8·0 $\times$ 10^8 counts/min per μM.

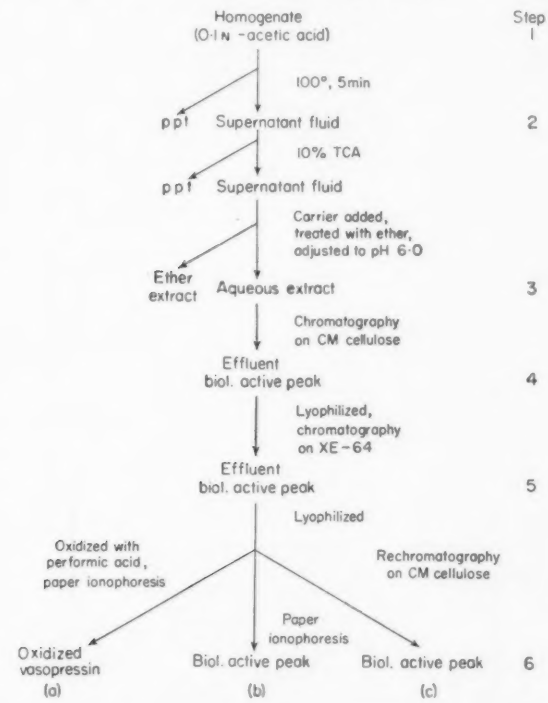


FIG. 1.

Isolation of unlabelled and ^{35}*S-labelled vasopressin.* Practically all of the steps employed in the isolation of vasopressin from the hypothalamus and neurohypophysis of the dog (cf. flow sheet, Fig. 1) are based on observations from a number of laboratories concerned with the chemistry and chromatographic properties of vasopressin (ACHER *et al.*, 1952, 1957, 1958; WARD and GUILLEMIN, 1957) which have been adapted to the special problem of isolating from these tissues minute quantities of labelled vasopressin in isotopically homogeneous form. The biological activity (total Units and Units/mg) was determined at each step (Table 1). After chromatography on XE-64 (Step 5) vasopressin was obtained having a biological potency of about 300–350 USP Units/mg (mg refers to mg of protein measured by the Folin procedure with bovine serum albumin as standard). It was this material which was taken through further purification steps in the labelling experiments to obtain vasopressin with constant specific activity (counts/min per Unit).

Since the hypothalamus and neurohypophysis of the dog contain a total of 5–50 μg of pressor material, it was essential to add carrier vasopressin at an early step (Step 3)

* Determined in collaboration with Dr. ARNOLD POWELL.

TABLE 1.—PURIFICATION OF VASOPRESSIN FROM HYPOTHALAMUS AND NEUROHYPOPHYSIS OF DOG

State of purification (cf. Methods and Fig. 1.)	Neurohypophysis		Hypothalamus	
	Total Units†	Units per mg protein	Total Units†	Units per mg protein
1	123*	5·0*	11·9*	0·03*
2	123 (110 to 139)	6·5	11·9 (10·3 to 13·8)	0·27
3	107 (99 to 115)	56·5	10·3 (9·4 to 11·1)	1·47
4	82 (78 to 86)	350	7·2 (5·8 to 9·0)	117
5	58 (53 to 64)	347	2·6 (2·1 to 3·1)	290
6C	22 (20·7 to 23·6)	385		

* Not actually determined but based on the assumption that the extraction of vasopressin into 0·1 N-acetic acid (Step 2) involved no losses; protein equals the sum of the protein soluble and insoluble in 0·1 N-acetic acid.

† Numbers in parentheses represent 95 per cent fiducial limits; total units have not been corrected for material removed at each preceding step for determination of protein.

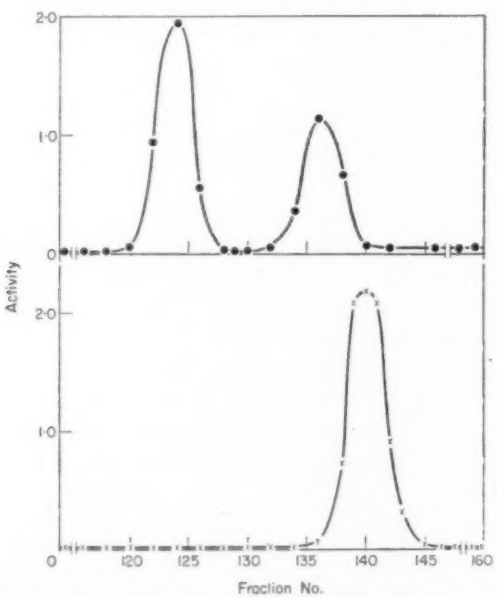


FIG. 2.—Chromatography on carboxymethyl cellulose (0·9 × 50 cm) of mixed samples of synthetic lysine and dog vasopressin (· ——— ·, upper portion); and highly purified arginine and dog vasopressin (× ——— ×, lower portion). Gradient elution started immediately and employed as described in the text; dog vasopressin was a partially purified sample (Table 1, Step 3); synthetic lysine vasopressin was kindly supplied by Dr. VINCENT DU VIGNEAUD. Ordinate plotted as total activity in terms of vasopressin units (see text).

to facilitate isolation and purification. At the present time there exist only two characterized mammalian vasopressin molecules; these being lysine (LVP) and arginine (AVP) vasopressin. As carrier in these experiments, highly purified AVP was used, after it had been established that dog vasopressin was separable from synthetic LVP and indistinguishable from AVP (Fig. 2). This finding that dog

vasopressin is identical with AVP is supported by the biological data of VAN DYKE (1959).

In Table 2, the specific activity data are given for pituitary and hypothalamic vasopressin after infusion of isotope for 16, 24 and 36 hr, and at each step of purification after and including XE-64 chromatography (Step 5). In the last 2 experiments (24 and 36 hr infusions) the biologically active peak obtained by rechromatography

TABLE 2.—SPECIFIC ACTIVITY OF LABELLED VASOPRESSIN

Expt. no.	Infusion (hr)	State of purification (cf. Fig. 1 and Table 1.)	Neuro-hypophysis (counts/min per Unit)	Hypothalamus (counts/min per Unit)	Ratio specific activity (H/N)
1	16	5	8·6	44·0‡	5·1
		6A	8·0	23·0	2·9
2	24	5	15·4	31·0	2·0
		6C fr 1	13·5		
		2	12·6	25·7	1·9
		3	15·0		
		6B	15·3	—	
		6A	†	†	
3	36	5	11·0	38·5	3·5
		6C fr 1	11·4		
		2	7·5	24·0	2·6
		3	9·0		
		6B	9·8	31·7	3·2

† Protein not measured but after paper ionophoresis the only recovered radio-activity was shown to be in an area corresponding to authentic oxidized vasopressin.

‡ In Expt. no. 1 Steps 4 and 5 were reversed in order, and the columns were about 2/3 the length of those used in Expt. no. 2 and 3. This procedure gave hypothalamic vasopressin (160–200 Units/mg) of an apparently lower degree of purity.

Expt. No. 1 infused continuously for 16 hr with ^{35}S-labelled *E. coli* protein hydrolysate containing a total of 5×10^9 counts/min; Expt. no. 2, $1{\cdot}0 \times 10^{10}$ counts/min; Expt. no. 3, $1{\cdot}2 \times 10^{10}$ counts/min.

on carboxymethyl cellulose (Step 6C) was divided into 3 emerging fractions (designated 1, 2, 3) whose specific activities were determined separately. Paper ionophoresis also gave material with similar specific activities. As a further criterion of isotopic purity (and in order to exclude non-specific S-S bound cysteine of high specific activity) the ^{35}S-labelled vasopressin was oxidized with performic acid to give oxidized vasopressin which now behaved as a neutral compound (paper ionophoresis, 0·05 M-pyridine acetate, pH 6·5) which moved a few cm towards the cathode (endosmosis). Under these conditions, unaltered vasopressin moved 12–14 cm towards the cathode, and cysteic acid moved far to the anode. The ratios of the specific activity of hypothalamic to neurohypophysial vasopressin ranged from 1·9 to 3·5 (exclusion of the ratio of 5·1 is explained in the legend to Table 2). In all probability, however, these ratios are maximal figures since the hypothalamic material could not be as extensively purified (because of the paucity of radioactive material) and also since the original estimates of pressor material in the hypothalamic extracts are unquestionably too low.

This result would, therefore, lead to an erroneously high dilution factor after addition of carrier vasopressin. This conclusion stems from the observation that hypothalamic extracts show considerable depressor activity (cf. VAN DYKE *et al.*, 1955) which would be expected to lead to low values of total pressor activity. When such extracts are passed through an anion exchange resin, part of the depressor material is removed and consistently higher values (20 per cent) are then obtained in the pressor assay (SACHS and WEINSTEIN, unpublished observations). However, such a correction has not been applied to the experiments in Table 2.

DISCUSSION

A study of vasopressin biosynthesis posed some serious experimental problems; these being: (1) the amount of vasopressin in an experimental animal as large as the dog is only about 5–50 μg; (2) the expected rate of synthesis in the dog is of the order of 0·01 μg per hour (SHANNON, 1942), necessitating the administration of isotopic precursors of high specific activity over extended periods of time; and (3) there are no definitive experiments at present concerning the site or sites of synthesis of this hormone. At the outset then, an attempt was made to answer essentially two questions. After the administration of highly labelled [^{35}S] cystine, could we effectively isolate from the hypothalamus and neurohypophysis of the dog labelled vasopressin in isotopically homogeneous form; and in so doing, could we also obtain some information bearing on the anatomical site(s) of synthesis? Although the former problem has been adequately resolved, the conclusions concerning the sites of synthesis of this neurohumor are equivocal.

The concept of neurosecretion implicates the supra optic and paraventricular nuclei (SCHARRER and SCHARRER, 1954) as the sites for the synthesis of vasopressin. This hormone is presumed to be bound to a carrier 'neurosecretory material' which is supposed to move in a protoplasmic flow via the supraopticohypophysial tract to the posterior lobe where storage and release occur. In the labelling experiments the ratios of the specific activity of hypothalamic to that of pituitary vasopressin were obtained after relatively short periods of continuous labelling (16–36 hr). These ratios ranged from 1·9 to 3·5 and on this basis the data obtained would appear to be inconsistent with any theory of neurosecretion that postulates a simple homogeneous two-compartment system (cf. SOLOMON, 1953). If it is assumed that the previously estimated rate of synthesis of vasopressin in the dog (SHANNON, 1942) of approximately 1–5 mU/hr is correct, the theoretically calculated ratios (ZILVERSMIT *et al.*, 1943) would be several hundred times the experimentally observed values. Nevertheless, these data do not preclude the hypothalamus as the site of the synthesis of vasopressin. Conceivably, labelled vasopressin, newly synthesized in the hypothalamus, might serve as the precursor of neurohypophysial vasopressin provided that this pool of labelled hormone represented only a small fraction of the hypothalamic vasopressin pool and that the equilibration of labelled vasopressin in the hypothalamus was slow compared to its transport into the neurohypophysis.

Obviously, the final answer must await a more detailed kinetic study as well as the elaboration of an *in vitro* system.

SUMMARY

A procedure was developed for the isolation of vasopressin of high purity from the hypothalamus and neurohypophysis of the dog. Subsequently, unanaesthetized

VOL.
5
1959/6

dogs were infused with [^{35}S] cystine (prepared from *E. coli* and with a specific activity of 7·0–8·0 $\times$ 10^8 counts/min per μM). After the infusion was complete the hypothalamus and neurohypophysis were each excised and used for the isolation of labelled vasopressin. Highly purified arginine vasopressin was used as carrier after it had been demonstrated that dog vasopressin was chromatographically separable from lysine vasopressin and indistinguishable from arginine vasopressin. Isotopic purity was established by rechromatography, paper ionophoresis, and performic acid oxidation and reisolation of oxidized vasopressin. In three experiments where dogs were infused continuously with [^{35}S] cystine for 16, 24, and 36 hr, the mean ratios of specific activity of hypothalamic to pituitary vasopressin were approximately 2·9, 2·0 and 3·1 respectively. These data would appear to be inconsistent with a theory of neurosecretion consisting of a simple homogeneous two-compartment system.

Acknowledgements—I would like to acknowledge the expert technical assistance of Mr. ROBERT HUGHES. Thanks are also due to Drs. BERNE, ROYCE and SAYERS of the Department of Physiology for their generous advice and assistance with some of the operative and bioassay procedures.

REFERENCES

ACHER R., CHAUVET J. and FROMAGEOT P. (1952) *Biochim. biophys. acta* **9,** 471.
ACHER R. and FROMAGEOT C. (1957) In *The Neurohypophysis* (Proceedings of the Colston Research Society) p. 39. Academic Press, New York.
ACHER R., LIGHT A. and DU VIGNEAUD V. (1958) *J. biol. Chem.* **233,** 116.
BARGMANN W., HANSTROM B. and SCHARRER E. (1958) *Zweites Internationales Symposium Uber Neurosekretion*, Springer-Verlag, Vienna.
COWIE D. B., BOLTON E. T. and SANDS M. K. (1952) *Arch Biochem. Biophys.* **35,** 140.
DU VIGNEAUD V. (1955) *Proc. 3rd Intern. Congr. Biochem. Brussels*, p. 49.
DU VIGNEAUD V. *et al.* (1953)
ELLIS S. and SIMPSON M. E. (1956) *J. biol. Chem.* 220, 939.
HELLER H. (1957) *The Neurohypophysis* (Proceedings of the Colston Research Society). Academic Press, New York.
HIRS C. H. W., MOORE S. and STEIN W. H. (1953) *J. biol. Chem.* **200,** 493.
HIRS C. H. W., MOORE S. and STEIN W. H. (1954) *J. Amer. chem. Soc.* **76,** 6063.
LOWRY O. H., ROSEBROUGH H. J., FARR A. L. and RANDALL R. J. (1951), *J. biol. Chem.* **193,** 265.
Pharmacopeia of U.S. (1955), XV, 776.
POPENOE E. A. and DU VIGNEAUD V. (1953) *J. biol. Chem.* **205,** 133.
ROBERTS R. B., ABELSON P. H., COWIE D. B., BOLTON E. T. and BRITTEN R. J. (1957) *Carnegie Inst. Wash.* Publication 607.
SACHS H. (1957) *J. biol. Chem.* **228,** 23.
SACHS H. (1959) *Biochim. biophys. acta* **34,** 572.
SCHARRER E. and SCHARRER B. (1954). *Recent Progr. Hormone Res.* **10,** 183.
SHANNON J. A. (1942) *J. exp. Med.* **76,** 387.
SLOPER J. C. (1958) *Int. Rev. Cytol.* **7,** 337.
SOLOMON A. K. (1953) *Advanc. biol. med. Phys.* **3,** 65.
VAN DYKE H. B. (1959) *XXI Int. Congr. Physiol. Sciences, Buenos Aires*, p.61.
VAN DYKE H. B., ADAMSON K. JR. and ENGEL S. L. (1955) *Recent Progr. Hormone Res.* **11,** 1.
WARD D. M. and GUILLEMIN R. (1957) *Proc. Soc. exp. Biol., N.Y.* **96,** 568.
ZILVERSMIT D. B., ENTENMAN C. and FISHLER M. C. (1943) *J. gen. Physiol.* **26,** 325.

Journal of Neurochemistry, 1960, Vol. 5, p. 304 to 306. Pergamon Press Ltd. Printed in Northern Ireland

PRELIMINARY NOTE

The effect of flavin adenine dinucleotide on the electroencephalogram modified by chlorpromazine

(*Received* 3 *November* 1959)

In an earlier study it was found that chlorpromazine inhibited D-amino acid oxidase. The experimental results obtained by kinetic study using a purified oxidase system showed that the chlorpromazine competed with the flavin adenine dinucleotide (FAD) for the enzyme protein, and the dissociation constant of chlorpromazine–oxidase–protein complex was found to be $2{\cdot}3 \times 10^{-5}$ M (Yagi, Nagatsu and Ozawa, 1956). Recently, it has been found that chlorpromazine at higher concentration than 10^{-5} M, which was used in the above-mentioned experiment, inhibited the fluorescence of FAD as a result of direct complex formation of both substances (Yagi, Ozawa and Nagatsu, 1959). Accordingly, it was thought that FAD might decrease the action of chlorpromazine *in vivo* by these two mechanisms, i.e. competition and complex formation. The antagonistic action of FAD and riboflavin-5′-monosulphate (FMS) found in the D-amino acid oxidase system (Egami and Yagi, 1956) was also reflected in functional phenomena of the brain, such as the electroencephalogram (EEG) (Muramatsu, Ando, Nagatsu and Yagi, 1958), and some interesting relationships between the chemical structures of the various flavin derivatives and their influence on the EEG were noted (Muramatsu, Ando, Nagatsu and Yagi, 1959).

These results suggested that the actions of FAD and chlorpromazine are opposed to each other *in vivo*. The effect of FAD on the EEG modified by chlorpromazine was therefore studied from this point of view.

MATERIALS AND METHODS

Chlorpromazine was supplied by Rhône-Poulenc-Specia. FAD was prepared by the method of Yagi, Matsuoka, Kuyama and Tada (1956). The purity of this sample was above 92%, and it contained no other flavins, nucleic acids or metals. The recording of the EEG was carried out as described by Muramatsu, Ando, Nagatsu and Yagi (1958, 1959).

Guinea pigs were used for the experiments. To avoid any effects of anaesthesia which may be potentiated by chlorpromazine, the animals were not anaesthetized. After craniotomy, two types of electrode were applied to the brain: one was set on the surface of the cerebral cortex to record the electrocorticogram (ECoG), and the other was put into the thalamic region to record the deep EEG. The exact position of the deep electrode was checked by histological investigation of the brain after the experiment. The reference electrode was put on the frontal area of the cranium, and monopolar leads were taken between the reference electrode and the cortex or the thalamic region. A GRASS MODEL III-D Electroencephalograph was used for recording.

When a stable EEG was obtained, 1 ml of an aqueous solution of chlorpromazine (5 mg/ml) was injected intraabdominally ($2{\cdot}8 \times 10^{-5}$ moles/kg of animal), and the changes in the EEG were observed. When the most marked changes appeared, 1 ml of an aqueous solution of FAD (4 mg/ml) was injected intraabdominally ($1{\cdot}0 \times 10^{-5}$ moles/kg of animal), and the effects on the EEG were observed. At the same time, synchroscopic observation of the EEG was carried out. The synchroscope is a cathode ray oscilloscope designed to analyse the EEG more precisely than the routine pen-writing electroencephalograph which could not show the fine changes (Ando and Fujioka, 1955).

RESULTS

After the injection of chlorpromazine, the frequency of the EEG decreased, and high voltage irregular slow waves appeared continuously as shown in Fig. 1. A-B. Thirty min after the injection of

VOL.
5
1959/6

chlorpromazine, when the EEG change had become marked, the aqueous solution of FAD was injected. The frequency was increased by the injection of FAD, and the characteristic 10–15 cyc/sec high voltage activity in spindle form appeared periodically as shown in Fig. 1. B-D.

The synchroscopic observations of these changes in the EEG are shown in Fig. 2. The irregular slow waves after the injection of chlorpromazine were observed more clearly than in the routine

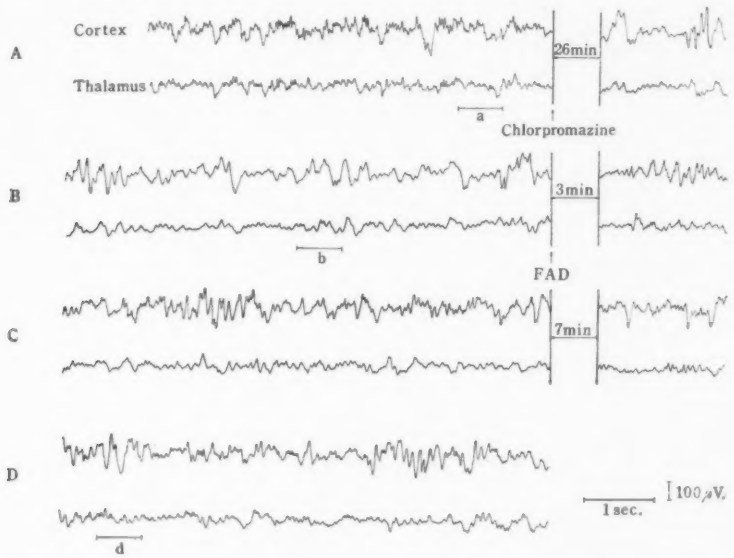


FIG. 1.—Effect of successive injection of chlorpromazine and FAD on ECoG and deep EEG.
(A) ECoG and deep EEG from the thalamus before and after the administration of chlorpromazine.
(B) Effect of the administration of FAD on the continued EEG of (A).
(C) The continued EEG of (B).
(D) The continued EEG of (C).

EEG; and these were reduced in number after the injection of FAD. The influence of FAD on the EEG changes induced by chlorpromazine continued for about 20 min, and then high voltage irregular slow waves appeared again. These changes in the EEG, recorded routinely or by synchroscopic observation, were found in each of 15 experimental animals.

DISCUSSION

The changes in the EEG after the administration of chlorpromazine agreed with other recent reports; high voltage irregular slow waves appeared (BRADLEY and HANCE, 1955). The administration of FAD partially reversed the effect of chlorpromazine on the EEG. This was seen more distinctly by synchroscopic analysis of the EEG.

As reported in an earlier paper (YAGI, NAGATSU and OZAWA, 1956), the dissociable flavin enzymes such as D-amino acid oxidase are inhibited by rather low concentrations (10^{-5} M) of chlorpromazine, which competes with FAD for the apoenzyme. It would therefore appear that chlorpromazine may inhibit that part of the oxidation in the brain which is mediated by the dissociable flavin-containing enzymes. This might have some relation to the modification of cerebral activity by chlorpromazine and its partial reversal by FAD. Direct complex formation between FAD and chlorpromazine (above 10^{-5} M) might reduce the effective level of chlorpromazine *in vivo*. The reversing action of FAD on the EEG modified by chlorpromazine may be explained by these two mechanisms; competition in the brain and complex formation *in vivo*.

The action of FAD on the EEG modified by chlorpromazine was not so completely competitive

as on the EEG modified by FMS. This may be explained by the fact that FMS is a specific inhibitor of flavin-containing enzymes, whereas chlorpromazine inhibits noncompetitively many enzymes in the brain other than flavin-containing enzymes: hexokinase, tricarboxylic acid cycle enzymes, cytochromes (BERNSOHN, NAMAJUSKA, and COCHRANE, 1956), and oxidative phosphorylation (ABOOD, 1955; DAWKINS, JUDAH and REES, 1958, 1959).

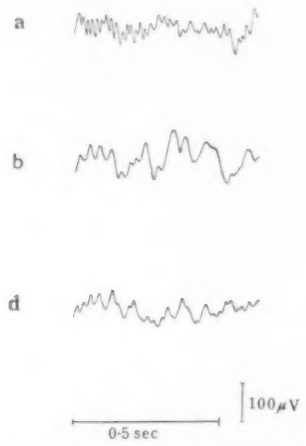


FIG. 2.—Synchroscopic observation of deep EEG from the thalamus. (a), (b) and (d) correspond to the parts (a), (b) and (d) in (A), (B) and (D) of Fig. 1.

From the EEG observations, it may be concluded that the injection of a large amount of FAD diminishes the effect of chlorpromazine on the brain. This suggests that FAD might be used to counteract the effects of an overdose of chlorpromazine.

SUMMARY

FAD partially reverses the effects of chlorpromazine on the EEG. This was observed in the ECoG and the deep EEG recorded by routine electroencephalography and seen by synchroscopic analysis. The reversing action of FAD may be attributed to competition for an enzyme protein and to the formation of a complex *in vivo*.

Department of Biochemistry
KUNIO YAGI
TAKAYUKI OZAWA

Department of Biochemistry, and
Department of Neuropsychiatry
School of Medicine
Nagoya University, Japan
MORIAKI ANDO
TOSHIHARU NAGATSU

REFERENCES

ABOOD L. G. (1955) *Proc. Soc. Exp. Biol. N.Y.* **88,** 688.
ANDO M. and FUJIOKA K. (1955) *Folia psychiat. neur. jap.* **9,** 264.
BERNSOHN J., NAMAJUSKA I. and COCHRANE L. S. G. (1956) *Arch. Biochem. Biophys.* **62,** 274.
BRADLEY P. B. and HANCE A. J. (1955) *J. Physiol.* **129,** 50 P.
DAWKINS M. J. R., JUDAH J. D. and REES K. R. (1958) *Nature, Lond.* **182,** 875.
DAWKINS M. J. R., JUDAH J. D. and REES K. R. (1959) *Biochem. J.* **72,** 204.
EGAMI F. and YAGI K. (1956) *J. Biochem. Tokyo* **43,** 153.
MURAMATSU T., ANDO M., NAGATSU T. and YAGI K. (1958) *Nature, Lond.* **182,** 457.
MURAMATSU T., ANDO M., NAGATSU T. and YAGI K. (1959) *J. Neurochem.* **4,** 229.
YAGI K., MATSUOKA Y., KUYAMA S. and TADA M. (1956) *J. Biochem. Tokyo* **43,** 93.
YAGI K., NAGATSU T. and OZAWA T. (1956) *Nature, Lond.* **177,** 891.
YAGI K., OZAWA T. and NAGATSU T. (1959) *Nature, Lond.* **184** 982

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 307–310. Pergamon Press Ltd. Printed in Northern Ireland

THE NON-HAEMIN IRON IN THE CEREBRAL CORTEX IN ALZHEIMER'S DISEASE

Bo Hallgren and Patrick Sourander
Institute of Medical Chemistry and Institute of Pathology
University of Gothenburg, Sweden

(*Received* 25 *November* 1959)

In a previous study (Hallgren and Sourander, 1958) we determined the content of non-haemin iron in different parts of the human brain from birth to old age. The distribution of the non-haemin iron in the cerebral cortex showed a characteristic

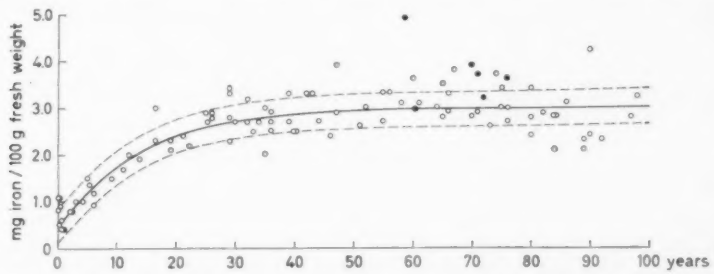


Fig. 1.—Non-haemin iron in the prefrontal cortex. The open circles in Figs. 1 and 2 represent normal cases and the filled circles cases of Alzheimer's disease. The calculated regression line for the normal cases has been drawn unbroken, the dotted lines denote the S.E. of estimate.

pattern. The highest concentrations were found in the motor cortex, lower concentrations being present in the occipital, sensory and parietal cortex in that order. The lowest figures were obtained for the prefrontal and temporal cortex. An increase in

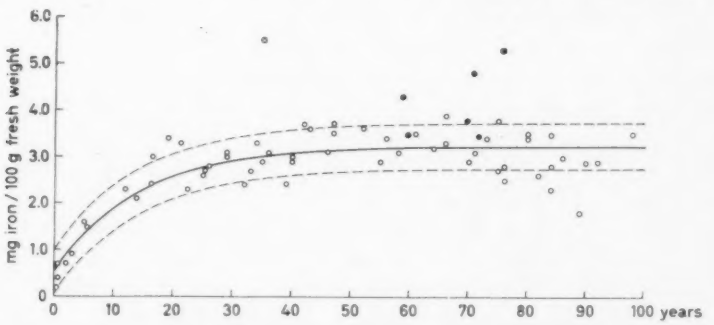


Fig. 2.—Non-haemin iron in the temporal cortex. For explanation see Fig. 1.

non-haemin iron occurred with advancing age in all regions of the brain examined except the medulla oblongata. The rise in iron content was rapid during the first two decades of life and then more gradual. The relation between the non-haemin iron content of the prefrontal cortex and the age is demonstrated in Fig. 1 and of the temporal cortex in Fig. 2. In young and middle aged individuals no histologically

demonstrable iron could be found in the cerebral cortex. Minimal iron deposits were detected in the motor and occipital cortex after the age of about 65 years.

In two brains and one biopsy specimen from cases of Alzheimer's disease, GOODMAN (1953), using the Prussian blue reaction, revealed the presence of large amounts of substance staining for iron in cells which had undergone neurofibrillary degeneration, within argentaffine plaques, in the oligodendroglia, in microglia and in one case in the walls of the blood vessels. GOODMAN suggested that Alzheimer's disease might be due to a disturbance in the metabolism of iron of the brain. Hitherto no quantitative estimations of the non-haemin iron of the brain have been performed in Alzheimer's disease. In order to decide whether the histologically demonstrable iron is due to a real increase in the non-haemin iron content it is necessary to include quantitative chemical determinations.

MATERIAL AND METHODS

The brains of 6 patients suffering from Alzheimer's disease were investigated; 5 from Lillhagens hospital, Gothenburg, and 1 from Ullerâkers hospital, Uppsala. The specimens were taken from different parts of the cerebral cortex and the extrapyramidal system.

Histological methods. For histological demonstration of the non-haemin iron the Turnbull blue method was used. The specimens from the fresh brains were fixed in 96% ethanol, embedded in paraffin and cut at 20 μ. For diagnostic purposes the toluidine blue staining method for nerve cells, PALMGREN's (1948) silver method for fibrillary degeneration of nerve cells and argentaffine plaques and HOLZER's (1921) stain for astroglia cells were used. The patho-anatomical diagnosis of Alzheimer's disease was based on the occurrence of large numbers of argentaffine plaques and Alzheimer's fibrillary changes in the atrophic cortex of the brain.

Chemical methods. Using unfixed material the non-haemin iron was determined by the method developed by HALLGREN (1953), the iron content being calculated on a fresh weight basis. The dry weight of the samples was also determined in order to exclude cases of oedema of the brain.

RESULTS

Histological findings. The characteristic features, argentaffine plaques and fibrillary changes, of Alzheimer's disease were most prominent in the frontal and temporal cortex. Histologically demonstrable iron was regularly found in fairly large amounts in these regions (see Table 1). Up to the present we have not been able to find iron in histological sections from the frontal and temporal cortex of normal brains (HALLGREN and SOURANDER, 1958). Considerable amounts of iron were detected in the motor and occipital cortex in the cases of Alzheimer's disease. In normal brains of old people minimal amounts of iron were found in the motor and occipital cortex (GELLERSTEDT, 1933; HALLGREN and SOURANDER, 1958). Most of the histologically visible iron was localized in astrocytes and microglia cells often surrounding the plaques (Figs. 3–5). Occasionally iron granules were seen also in nerve cells.

Chemical determinations of non-haemin iron. As can be seen in Fig. 1, 5 of the 6 cases of Alzheimer's disease show normal non-haemin iron values in the prefrontal cortex. The sixth case, a woman aged 59 years, showed a high iron content in the prefrontal, precentral, postcentral and parietal cortex. There was, however, slight haemolysis in this case which might have influenced the iron determinations. In the temporal cortex (Fig. 2) the values for 4 cases fall within the normal range and 1 case showed an elevated value. This patient, a woman who died at 76 years of age, had cerebral arteriosclerosis as well as Alzheimer's disease. One case falls close to the

VOL. 5 1959/6

OL.
5
9/60

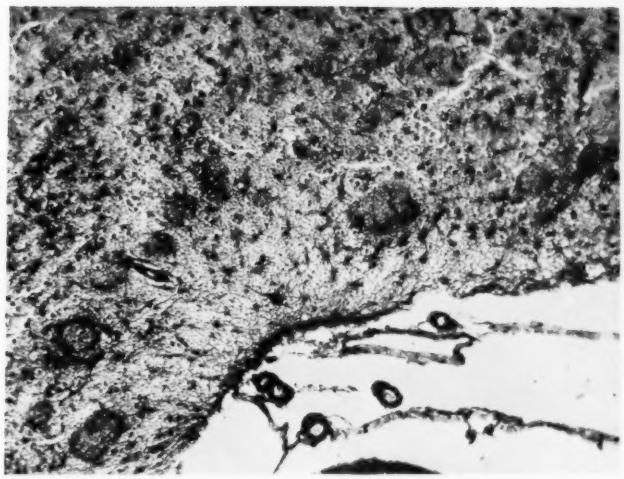


FIG. 3.—Temporal cortex showing numerous plaques surrounded by large astrocytes. Case of Alzheimer's disease. HOLZER's glia stain. ×200.

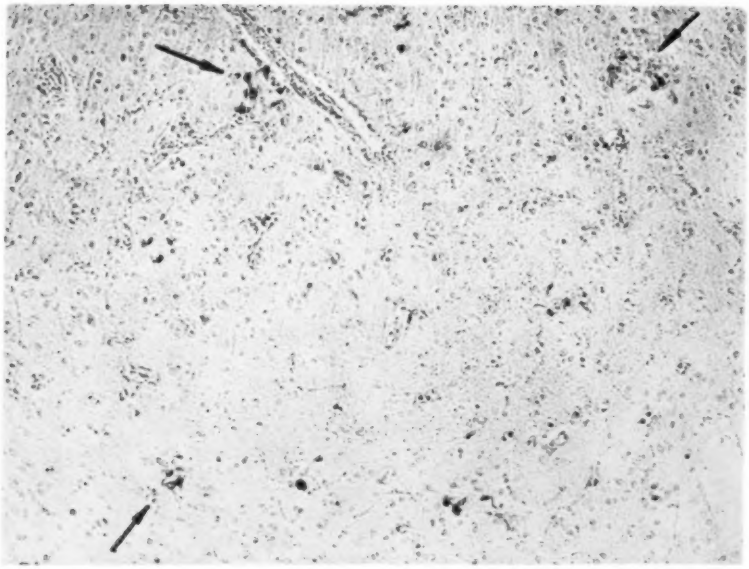


FIG. 4.—Groups of iron containing glial cells in the cortex (three of them indicated by arrows). Case of Alzheimer's disease. Turnbull blue method. × 145.

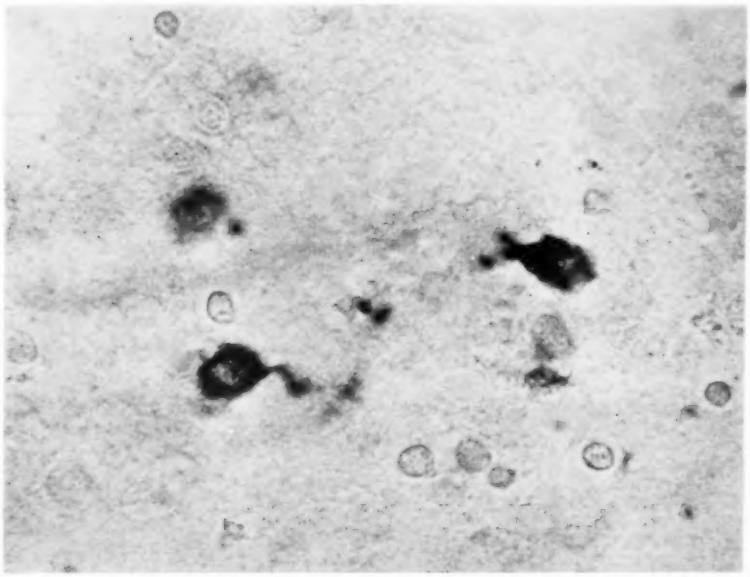


FIG. 5.—A group of glial cells containing iron granules in the periphery of a plaque. Case of Alzheimer's disease. Turnbull blue method. ×885.

VOL.
5
1959/6

upper normal distribution limit ($M + 3\sigma$). In the precentral, postcentral, parietal and occipital cortex normal values were found except in the brain from the 59-year-old woman discussed above. There does not seem to be any noteworthy increase in non-haemin iron content in the cerebral cortex in Alzheimer's disease. A slight increase in

TABLE 1.—HISTOLOGICAL FINDINGS AND CHEMICAL DETERMINATIONS OF THE NON-HAEMIN IRON IN SIX CASES OF ALZHEIMER'S DISEASE

Age (years)	Duration of disease (years)	Brain weight (g)	Histological findings						Non-haemin iron (mg/100 g fresh wt)	
			Plaques		Fibrillary changes		Turnbull blue reaction			
			PC	TC	PC	TC	PC	TC	PC	TC
59	2	1130	+	++	+	+++	+++	+++	4·9	4·3
61	8	1150	+++	+++	+++	+++	+	+++	3·0	3·5
70	4	1000	+++	+++	+++	+++	++	+++	3·9	3·8
71	10	1250	+++	+++	+++	+++	++	++	3·7	4·8
72	10	1080	+++	+++	+++	+++	++	+	3·2	3·4
76	11	1000	+++	++	+++	++	++	+++	3·3	5·3

PC = prefrontal cortex
TC = temporal cortex
Few plaques +; plaques in every field ++; large number of plaques in every field (see Fig. 3) +++.
The same type of classification has been used for the occurrence of fibrillary changes in nerve cells.
Iron granules in occasional glial cells +
Iron granules in a few glial cells ++
Iron granules in glial cells of every field +++

iron content calculated on a weight basis could be a consequence of the atrophy of the cortex, assuming that no iron had disappeared. Normal iron values were found in the extrapyramidal system.

DISCUSSION

The occurrence of histologically demonstrable iron in the frontal and temporal cortex is a very characteristic feature in Alzheimer's disease, not only in acute cases as shown by GOODMAN (1953), but also in cases of long duration. In 6 cases of Alzheimer's disease which we have investigated an increase of stainable iron was regularly found in different cortical regions. No chemical determinations of the non-haemin iron of the brain in Alzheimer's disease have hitherto been performed. In the 6 cases examined by us a normal iron content was found in nearly all specimens examined. Thus there is a discrepancy between the increase in stainable iron found in histological sections and the normal values for the non-haemin iron determined by chemical methods. It seems likely that the altered staining properties of the non-haemin iron in Alzheimer's disease are due to a redistribution of iron caused by changes in the binding of the iron in the brain tissue. GOODMAN (1953) suggested that Alzheimer's disease might be due to a primary disturbance in the cerebral metabolism of iron. Our results do not support this hypothesis.

SUMMARY

1. The non-haemin iron in the brain has been studied in 6 cases of Alzheimer's disease.

2. In the cerebral cortex a large increase in stainable iron is found. The Turnbull blue method was used.

3. Quantitative chemical determinations of the non-haemin iron in different cortical areas revealed values in the normal range.

4. It is suggested that the increase in histologically demonstrable iron is due to an altered binding of the iron in the brain tissue.

Acknowledgements—The financial support of Statens Medicinska Forskningsråd is gratefully acknowledged. We are indebted to Dr. HAKON SJÖGREN for the thorough clinical examination of the cases from Lillhagens Hospital.

REFERENCES

GELLERSTEDT N. (1933) *Uppsala LäkFören. Förh.* **38**, 103.
GOODMAN L. (1953) *J. nerv. ment. Dis.* **118**, 97.
HALLGREN B. (1953) *Uppsala LäkFören. Förh.* **59**, 79.
HALLGREN B. and SOURANDER P. (1958) *J. Neurochem.* **3**, 41.
HOLZER, W. (1921) Cited by B. ROMEIS: *Mikroskopische Technik* (München) 1948 p. 432.
PALMGREN A. (1948) *Acta zool., Stockh.* **29**, 377.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 311–322. Pergamon Press Ltd. Printed in Northern Ireland

THE INCORPORATION OF ^{14}C DERIVED FROM GLUCOSE INTO THE PROTEINS OF THE BRAIN CORTEX, AT REST AND DURING ACTIVITY*

A. GEIGER, N. HORVATH and Y. KAWAKITA
Research Laboratories, Department of Psychiatry
University of Illinois College of Medicine Chicago, Illinois

(*Received* 2 *October* 1958)

^{14}C GLUCOSE added to the perfusion blood is rapidly incorporated into the free amino acids of the brain, and, at a slower rate, into its structural components, as shown in the preceding paper (BARKULIS *et al.*, 1960). The incorporation of ^{14}C glucose into amino acids by brain slices has been demonstrated previously by BELOFF-CHAIN *et al.* (1955), and that of labelled amino acids into brain cell proteins *in vivo* by WAELSCH (1957), GAITONDE and RICHTER (1955, 1956), and PALLADIN *et al.* (1957). This question was examined in brain perfusion experiments by BARKULIS *et al.* (1960). In the present paper, the effects of convulsive activity on incorporation rates of ^{14}C from glucose into different cortical areas and into proteins of various cell fractions was studied at rest and during and after convulsive activity induced by metrazol or by electric stimulation.

The data presented are the result of 11 perfusion experiments. The time course of the ^{14}C incorporation into the brain cortex was followed on samples excised at various time intervals. One or two resting brain samples were taken before convulsions were induced. In several preliminary experiments, simultaneously excised brain samples were analysed and compared in order to determine the feasibility of following the time course of the ^{14}C incorporation on cortical samples taken from different areas.

The brain perfusion method and the use of a simplified blood of known composition made it possible to standardize the experimental conditions, to maintain these constant throughout the experiments, and to preserve a number of the physiological functions of the brain. Any exchange of chemical compounds between the brain and other organs of the body was excluded. This arrangement made it possible also to determine the extent of the *in vivo* synthesis of amino acids in the brain and to maintain in the brain a constant pool of labelled precursors for protein synthesis. The former consideration was important in view of the rapid rates of exchange between the amino acids of the blood and of the brain, as shown by LAJTHA *et al.* (1957).

EXPERIMENTAL

The technique of perfusion has been previously described by GEIGER and MAGNES (1947), and GEIGER (1958). In the present experiments, 70–100 μC of uniformly ^{14}C-labelled glucose, dissolved in 2–4 ml of Ringer's solution, were added to 500–800 ml of the circulating simplified blood (GEIGER, 1958) 20–25 min after the perfusion was begun, at a time when the rate of oxygen consumption of the brain became steady and the physiological activity and responses of the brain normal. In order

* This work was supported by a grant (B-413) from the National Institute of Nervous Diseases and Blindness, National Institutes of Health. A preliminary report of some of the data has been presented (GEIGER *et al.*, 1958).

to allow proper mixing of the labelled glucose with the blood, zero time was taken after the lapse of 5 min following the addition of ^{14}C-labelled glucose to the blood reservoir. The blood usually contained 40–60 mg of carrier glucose per 100 ml. The blood consisted of cooled, defibrinated, and filtered ox blood which was washed in the centrifuge with a Krebs–Ringer solution and suspended in about 10 vol. of Krebs–Ringer solution for 1–2 hr in the cold room, centrifuged, and suspended once again. After 2 hr, the erythrocytes were centrifuged again and placed in 0·5 in. cellophane sausage casings, which were tied and suspended in a large container of Krebs–Ringer solution in the cold room. With frequent changes of the Ringer solution, the erythrocytes were dialysed for at least 2 days before use. To the Ringer solution, as well as to the erythrocytes, penicillin and streptomycin were added. These drugs were washed from the blood before the experiments, as excessive amounts induce convulsions. The perfusion blood contained 30% of the dialysed erythrocytes suspended in Krebs–Ringer solution containing 7% native bovine serum albumin (Pentex). The albumin had been passed through cation and anion exchange columns before use, and, in some instances, dialysed. The 'simplified' blood was adjusted to pH 7·3 with isotonic $NaHCO_3$ solution.

The blood flow rates were kept at 120–130 ml/min per 100 g brain, with a mean perfusion pressure of 80 mm Hg. The oxygen consumption, which was recorded continuously together with the blood flow, was usually between 5 and 6 ml of oxygen/min per 100 g brain at rest. Electrocorticograms from both hemispheres were recorded continuously, as well as pupillary and corneal reflexes and the reactions of the EEG to touch, or to pinching of toes. Only those experiments were used in which, according to these criteria, brain functions were normal. At stated intervals, brain cortex slices, 2–2·5 mm thick and weighing between 0·5 and 0·8 g, were excised from different cortical areas, frozen on dry ice, weighed, and stored, each individually, in stoppered vials until analysed. The exposed brain surface was covered with 'parafilm' and gauze. The exposed part of each hemisphere was roughly divided into frontal, parietal, and occipital areas between about 2 mm in front of the *crutiate sulcus* and the *occipital pole*. The slices were re-weighed before analysis to correct for any loss of water, homogenized in 9 volumes of 8% sucrose solution for 5 min, and subjected in the usual way to fractionation by differential centrifugation. The nuclei and cell debris were separated by centrifugation for 10 min at 600 **g** and the mitochondria by centrifugation for 30 min at 14,000 **g**. The fluffy loose precipitate on top of the mitochondria was decanted, together with the supernatant fluid, and re-centrifuged for another 30 min at 14,000 **g**. This sediment was called the intermediate fraction. The supernatant fluid, decanted from the intermediate fraction was centrifuged for 60 min at 100,000 **g** to yield the microsome fraction. The decanted supernatant fluid was brought to pH 5·2 by 0·05 N-HCl. The resulting suspension was centrifuged to yield the 'pH 5·2 fraction,' and the supernatant or 'albumin' fraction. Each fraction obtained from the sucrose fractionation was treated with about 10 ml of 10% TCA and the precipitate was washed twice with the same amount of 5% TCA. In one group of experiments, the precipitates were heated for 20 min at 92° after removing the last TCA wash, as described by SACHS (1957). The TCA washes were followed, according to SCHNEIDER (1945), by three washes with 10 ml each time of heated alcohol–ether (3 : 1) and hot TCA. The residue was dissolved in 1·5–2·5 ml of 0·5 N-NaOH. Portions of the NaOH solution were plated for counting with a 'Micromil' window flow counter of adequate sensitivity, and others digested for nitrogen determination by the Kjeldahl method. The radioactivity of the ^{14}C in the acid and lipid extracts were determined in most experiments. In some instances, it was not possible to obtain an absolutely clear supernatant fluid from the first TCA precipitate. Some loss of protein may have been incurred in this way.

RESULTS

The incorporation rates of ^{14}C into brain proteins in the Tables and Figures are related either to 1 mg of protein nitrogen (obtained by triplicate Kjeldahl analysis of each cellular fraction) or to 1 g (wet wt) of brain cortex. Altogether, 55 brain samples, derived from 11 perfusion experiments, were fractioned in sucrose and analysed. These perfusion experiments were supplemented by 12 analyses of brain samples obtained from normal non-perfused cats 60 min after an intraperitoneal injection of uniformly ^{14}C-labelled glucose.

The protein nitrogen data (Tables 1 and 2) were obtained by adding the amounts in each fraction. These values (multiplied by the conventional 6·25) show an average

VOL.
5
1959/6

of 73 mg protein in 1 g wet brain cortex. This concentration is lower than are those generally reported. The values found by LOWRY *et al.* (1954), and by ROBINS *et al.* (1957), are about 30 per cent higher. In part of the brain samples, the TCA residue was heated on the water bath as recommended by SACHS (1957). In others this heating was omitted but the samples were heated to 50° in alcohol–ether (3 : 1). The differences

TABLE 1.—PROTEIN NITROGEN CONTENT OF BRAIN CORTEX AT DIFFERENT TIMES OF PERFUSION

The values are means derived from analyses of 55 samples obtained during 11 perfusion experiments.

Duration of perfusion (min)	Protein nitrogen (mg/g brain cortex)
15	11·80 ± 0·6*
30	11·61 ± 0·57
45	11·46 ± 0·41
60	11·77 ± 0·51
75	12·11 ± 0·71
90	12·31 ± 0·33
	Mean 11·67

* Standard error of the mean.

TABLE 2.—DISTRIBUTION OF PROTEIN NITROGEN AMONG CELL FRACTIONS OF THE BRAIN CORTEX

The brain cortex was frozen immediately after excision and kept frozen for days prior to homogenization.

Cell fraction	Protein nitrogen (mg/g wet brain)	Percentage of total (%)
Nuclei	1·09 ± 0·07*	9·4 ± 2·0*
Mitochondria and intermediate	6·26 ± 0·17	53·9 ± 3·3
Microsomes	1·18 ± 0·06	10·2 ± 1·0
Proteins precipitated from supernatant fluid at pH 5·2	2·02 ± 0·19	17·4 ± 1·7
Proteins of supernatant fluid soluble at pH 5·2	1·12 ± 0·06	9·7 ± 1·1

* Standard error of the mean

between these two ways of extraction were very small. Loss of lipoproteins was not likely since lipids were extracted with alcohol and ether. A possible loss of proteins during fractionation and extraction may be responsible for the low results. Practically identical nitrogen concentrations were obtained in the cortex of perfused and normal, non-perfused brains with identical methods of fractionation and extraction. A slight increase in the protein content of the brain cortex is noticeable after 75 min of perfusion. This was probably due to a slight loss of water. It happened consistently in spite of precautions taken against drying of the surface and strict attention to the isotonicity of the perfusing blood.

In order to be able to follow the course of incorporation of ^{14}C glucose into the brain proteins on the same animal, it had to be ascertained whether or not brain cortex samples taken at different time intervals from different areas of the cortex are representative of the whole cortex. In a number of experiments, two or more different cortical areas were excised simultaneously at progressive stages of perfusion. Each brain cortex slice was homogenized in water. Portions of the homogenates were treated with TCA and with the extraction procedure described above. The results (Table 3), show that even after 30 min of perfusion, when the amounts of radioactivity incorporated into the proteins are still relatively low, the variations are small.

TABLE 3.—SPECIFIC ACTIVITY OF PROTEINS IN BRAIN CORTEX TAKEN SIMULTANEOUSLY FROM DIFFERENT AREAS

All values are counts/min per mg protein nitrogen.

Area*	Normal (not perfused)	Experiments with 30 min perfusion				Experiments with 60 min perfusion	
		Jan. 15	Feb. 6	Apr. 3	Apr. 23	Jan. 10	Mar. 12
L.F.							
L.P.	69	102	224	262	227	171	
L.O.		94			228		450
R.F.	66		212			191	
R.P.				270			
R.O.							438
L.O. + R.O.						168	

* Abbreviations: L.F. = left frontal; R.F. = right frontal; L.P. = left parietal; R.P. = right parietal; L.O. = left occipital; R.O. = right occipital.

The good agreement among different cortical areas taken simultaneously made it possible to follow the time-course of ^{14}C incorporation by taking biopsy samples of various cortical areas at different times.

In the following experiments, one cortical area was excised at a stated perfusion time, first the left frontal, then the right frontal, parietal, etc., or 2 or 3 small slices of about 0·2 g each, taken from each hemisphere simultaneously, were mixed in each sample.

The resting experiments (Fig. 1 and Table 4) show that the incorporation rates of ^{14}C into proteins are slightly, but progressively slowing down after 30 min of perfusion. This may be due to the conditions of the perfusion, or it may occur under natural conditions. A comparison of the perfused brain with that of the normal, awake, non-perfused cat shows that the incorporation rates in the perfused brain are rather higher. A slow drop of the rates of incorporation of labelled amino acids into brain proteins with time has been shown by WAELSCH *et al.* (1958) in normal rats.

The effect of convulsions, whether elicited by metrazol or by electric stimulation is characteristic and striking. During the convulsions, the incorporation rate of ^{14}C derived from glucose into brain proteins is greatly increased (Table 5, Fig. 1 and 3). Immediately after the convulsions, for about 15 min, the incorporation seems to come to a standstill, and even loss of radioactive proteins occurs in spite of the continued presence of [^{14}C] glucose in the circulating blood. After this lag period the rate of ^{14}C incorporation again increased, greatly surpassing that observed during the

VOL. 5 1959/6

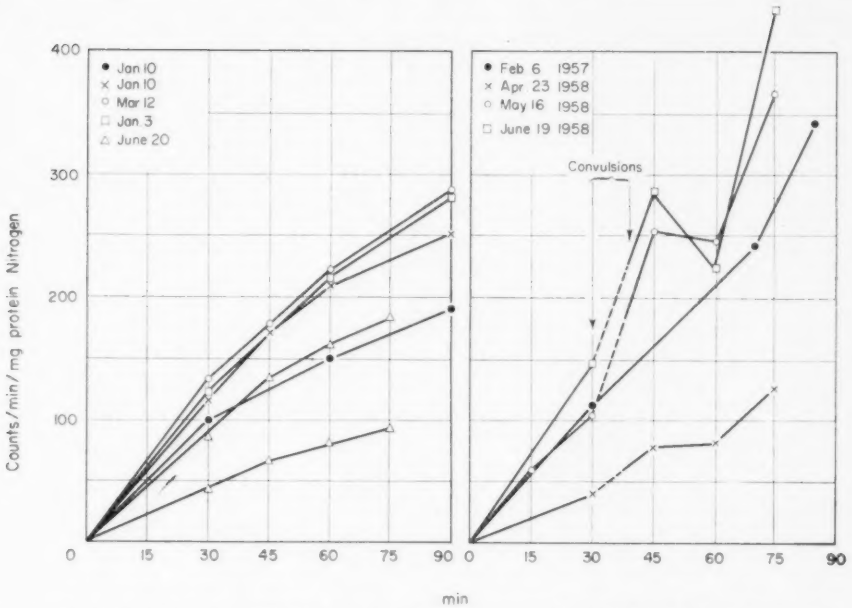


FIG. 1.—Incorporation of ^{14}C derived from glucose into proteins of brain cortex with increasing time (in min) of perfusion. Experiments under resting conditions at the left. In the experiments shown by the three upper curves at the right convulsions were induced by pentamethylenetetrazol; in the upper two at 30 min after addition of [^{14}C] glucose (dotted parts), and in the other at 70–75 min. In the experiment shown by the lowest curve at the right, the brain was stimulated (dotted part) through the frontal poles by a 12 volt, alternating current of 75 cycles of 0·1 msec duration for 3 min.

TABLE 4.—RATES OF INCORPORATION OF ^{14}C DERIVED FROM GLUCOSE INTO PROTEINS OF THE RESTING BRAIN CORTEX

The rates are calculated for 15 min periods with those for the first 30 min taken as unity and those for subsequent 15 min periods as percentages of the 30 min values.

Experiment	Period (min of perfusion)			
	0–30 (counts/min per 15 min per mg protein nitrogen)	30–45 (%)	45–60 (%)	75–90 (%)
Jan. 10 (P.P.)	58	95	69	42
Jan. 10 (F.F.)	50	60	60	60
Mar. 12	66	71	71	53
Jan, 31, 1957	62	—	76	53
Mean		75·5	71·5	52

previous periods. Thus, 40 min or more after the convulsions, 2 to 4 times as much ^{14}C was incorporated into the post-convulsive brains as into a resting brain during a comparable period of time.

In the next experiments the time-course of incorporation of [^{14}C] glucose into proteins of different cell fractions was measured. Uniformly reproducible separations

TABLE 5.—RATES OF INCORPORATION OF ^{14}C DERIVED FROM GLUCOSE INTO PROTEINS OF BRAIN CORTEX AS AFFECTED BY CONVULSIONS

Calculations as described in Table 4

Experiment	Period (min of perfusion)				
	0–30 (counts/min per 15 min per mg protein nitrogen)	30–45 (convulsions) (%)	45–60* (%)	60–75* (%)	75–90* (%)
May 16, 1958	52	304	−17	236	
June 10, 1958	72	200	−83	686	
Feb. 6, 1957	55			50	186
Apr. 23, 1958	20	200	20	235	
Mean		242	−40	419	

* Post-convulsive during these periods except that convulsions occurred during the 60–75 min period in the Feb. 6, 1957 experiment.

VOL. 5 1959/6

were obtained. Each cell fraction (derived usually from 0·5–0·8 g of brain) was treated and washed as described above.

In preliminary experiments (Table 6) it was ascertained that the numbers of counts obtained in cell fractions of different cortical areas excised simultaneously are comparable within 5–7 per cent. This was confirmed from time to time in the course of these experiments.

TABLE 6.—DISTRIBUTION OF ^{14}C IN PROTEINS OF CELL FRACTIONS

Two cortical areas were excised simultaneously for comparison.
60 min perfusion; counts/min per mg protein nitrogen

	March 21, 1958 Experiment		Jan. 10, 1958 Experiment	
	L.F.*	L.P.	R.F.	R.P.
Nuclei	73	83	107	98
Mitochondria	45·5	51	59	61
Intermediate	63·5	67	122	137
Mitochondria and intermediate	50	54	71	78
Microsome	96	114	228	240
pH 5·2 Precipitate	60	65	76	74
Albumins	110	65	116	106

* Abbreviations as in Table 3.

The most active cell fractions after 30 minutes perfusion with [^{14}C] glucose were the microsomes (Fig. 2), followed by the 'intermediate fraction,' the nuclei, the soluble proteins not precipitable at pH 5·2, the soluble proteins precipitated at pH 5·2, and

the mitochondria, in this order. After 60–75 min of perfusion, this order changed with respect to nuclei and intermediate fraction, the former incorporating at a higher rate than the latter.

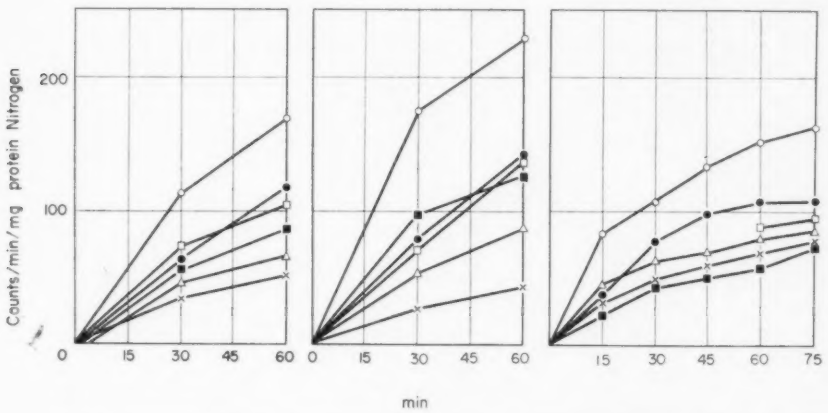


FIG. 2.—Incorporation of ^{14}C derived from glucose into the proteins of cellular components of the cortex. Three experiments are shown. The abscissae show the time in minutes from the addition of ^{14}C-labelled glucose to the perfusion blood. ○: microsomes; ●: Nuclei; □: intermediate fraction; ■: soluble proteins in supernatant fluid after precipitation at pH 5·2; △: proteins precipitated from supernatant fluid at pH 5·2; ×: mitochondria.

The characteristic changes in the rates of ^{14}C incorporation caused by convulsions (Fig. 1) are evident in each cellular fraction (Fig. 3). During the convulsions, the rate of incorporation of ^{14}C increased sharply in the microsomes, in the mitochondria, and in the pH 5·2 precipitable proteins of the supernatant fluid and possibly to a lesser extent in the other fractions. During the 15 min following the convulsions, there seemed to be no incorporation of ^{14}C into any of the cellular fractions, with the exception of the nuclei, where incorporation progressed at the previous rate. Some radioactivity was lost from most of the cell fractions, and especially from the microsomes. In two cases, the loss of ^{14}C from the microsomes amounted to over 30 per cent of their previous activity. During the 15 min following this lag period, the incorporation rates again increased very sharply so that at this stage the amounts incorporated into all cellular fractions were 3–4 times higher than incorporation rates at rest during a comparable period of time.

The loss of radioactivity from the microsomes and other cell fractions during the first 15 min of post-convulsive state may indicate that during this period a particular protein in the microsomes, with a high turnover rate, is being utilized and not re-synthesized. That this may be the case is indicated by experiments in which microsomal proteins were fractionated. All these experiments show that the amount of ^{14}C incorporated by brain proteins is sharply increased following activity.

A narcotized, but otherwise normal, non-perfused cat received an intraperitoneal injection of 100 μC of radioactive glucose. Sixty minutes later, it was killed in ether narcosis, and sections of its brain cortex were quickly removed and analysed. The results (Table 7) show the same incorporation pattern as did the resting perfusion experiments.

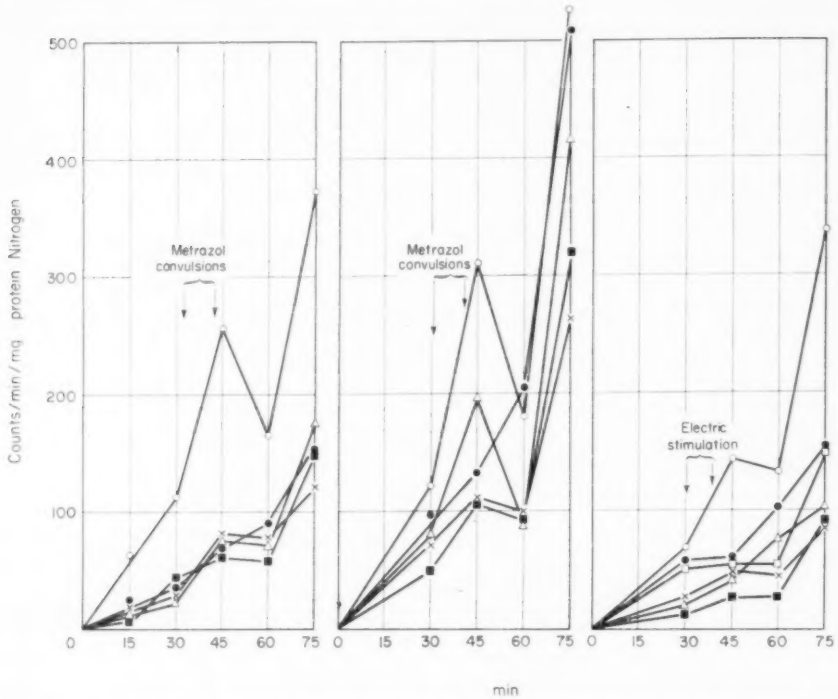


FIG. 3.—Effects of metrazol convulsions and of electric stimulation on the incorporation of ^{14}C derived from glucose into proteins of cellular components. Abscissae show time in min. Marking of curves as in Fig. 2.

TABLE 7.—DISTRIBUTION OF ^{14}C IN PROTEINS OF CELL FRACTIONS OF THE BRAIN IN A NON-PERFUSED, NARCOTIZED CAT, 60 MIN AFTER ADMINISTRATION OF 100 μC OF UNIFORMLY ^{14}C-LABELLED GLUCOSE

	Counts/min per mg protein nitrogen
Nuclei	78
Mitochondria	31
Intermediate fraction	62
Microsomes	121
pH 5·2 precipitate from supernatant fluid	58
pH 5·2 proteins remaining in solution after precipitation	37

In order to obtain a general picture of the relative distribution of the ^{14}C from glucose incorporated into proteins of different cell fractions of the brain cortex, the mean results obtained on 55 brain cortex samples in the foregoing experiments are given in Table 8. These values show that relatively the largest amounts of ^{14}C are found in the mitochondria, more during activity than during rest.

In every experiment there was an increase in the amount of mitochondrial protein after stimulation. For instance, in the experiments of May 16 and June 19, the mitochondrial protein nitrogen per gr of wet brain was 6·07 mg before stimulation,

6·70 mg 15 min after stimulation, and 7·30 mg 30 min after stimulation. This finding supports the results of R. S. GEIGER (1956), who found that mitochondria and possibly other granules agglomerated in living neurons cultured *in vitro*, during stimulation.

TABLE 8.—THE DISTRIBUTION OF ^{14}C GLUCOSE IN THE PROTEINS OF CELL FRACTIONS

The data are expressed as percentages of the total proteins of the brain cortex homogenate, which is taken as 100 per cent.

	Time of perfusion in minutes				
	15	30	45	60	75
NUCLEI					
Resting brain	8·0(±3)	11·1 (±1·5)		13·0 (±1·1)	
Stimulated brain			7·7 (±1·8)	13·1 (±2·3)	12·0 (±1·2)
MITOCHONDRIA					
Resting brain	41·4	29·0 (±6·4)		26·0 (±3·8)	
Stimulated brain			38·3 (±0·4)	39·0 (±2·1)	39·1 (±0·1)
INTERMEDIATE					
Resting brain		14·4 (±2·6)		13·2 (±1·1)	
Stimulated brain			10·2 (±3·7)	10·5 (±3·1)	8·6 (±3·7)
MICROSOMES					
Resting brain	38·7	21·0 (±3·1)		20·3 (±1·8)	
Stimulated brain			23·7 (±3·0)	16·6 (±1·7)	17·0 (±2·2)
CYTOPLASMIC SUPERNATANT FLUID					
A. *Protein Precipitate at* pH 5·2					
Resting Brain	11·7	15·7 (±2·4)		15·1 (±0·9)	
Stimulated brain			15·1 (±4·2)	15·1 (±2·6)	11·3 (±1·3)
B. *Protein remaining in solution*					
Resting brain		9·1 (±4·1)		10·7 (±3·2)	
Stimulated brain			11·3 (±4·1)	5·8 (±1·7)	7·0 (±1·8)

In 4 experiments, microsomes obtained from 1 to 2 g of brain cortex were resuspended and homogenized in 8 ml of 0·2 M-tris buffer, pH 8·6, with the addition of 8 mg of sodium glycocholate. This suspension was centrifuged at 35,000 **g** for 30 min and at 100,000 **g** for 60 min. The fraction coming down at 35,000 **g** contained most of the activity, if the counts were related to the nitrogen content. In a typical experiment the fraction, coming down at 35,000 **g** contained 12,500 counts/min per mg nitrogen; the second, at 100,000 **g**, 3200 counts/min per mg nitrogen; and the supernatant fluid 4200 counts/min per mg nitrogen. CLOUET and RICHTER (1959) recently extracted a protein showing a high turnover rate from microsomes of the brain.

Although the incorporation rates into the cortex of the frontal, parietal, and occipital areas are fairly similar, the rates of incorporation into other parts of the brain show wide variations. A good general idea of the rates at which the ^{14}C of glucose is incorporated into different anatomical structures of the brain can be obtained by radioautography of cross-sections of the brain. The results of such experiments which are now in progress will be reported elsewhere.

In order to ascertain that the radioactivity of the proteins represents incorporation of ^{14}C-labelled amino acids, proteins of two brain cortex samples obtained in two perfusion experiments were hydrolysed, the amino acids were separated by column chromatography on Dowex 50 and Dowex 1, and their average specific activities, as well as the specific activity of glutamic and aspartic acids, were determined. For these analyses, about 10 mg of brain proteins were hydrolysed in 6 N-HCl in a sealed glass tube for 40 hr at 100°. After filtration and dilution with water, the HCl and water were distilled off *in vacuo* with several additions of water. The amino acids were dissolved and put first on Dowex 50 and then on Dowex 1 columns and separated as described elsewhere for free amino acids (BARKULIS *et al.*, 1960).

In one experiment (June 19, 1958), after the usual extractions with cold TCA, alcohol–ether, hot TCA, and drying, the dry protein, dissolved in 0·5 N-NaOH, had an activity of 79 counts/min per mg protein after correction for self absorption. From the hydrolysate of 10 mg of dry protein, 57·8 μmoles of amino acid were separated, with a total activity of 627 counts/min. From this amino acid mixture, 7·6 μmoles of glutamic acid were isolated, with a specific activity of 34 counts/min per μmole and 3·3 μmoles of aspartic acid with a specific activity of 22 counts/min per μmole.

DISCUSSION

From the data contained in this paper, which show that carbon atoms derived from glucose are incorporated into proteins, and from the incorporation rates of ^{14}C into the amino acids (BARKULIS *et al.*, 1960), it is not possible to calculate turnover rates of proteins, because none of the cell fractions have yet been sufficiently fractionated to yield individual proteins. This work is in progress. In calculating the rate at which amino acid carbon is incorporated into proteins, the incorporation rates of ^{14}C from glucose into amino acids, have to be considered. These have to be multiplied by about 5 to obtain the amount of amino acid carbon incorporated because the amino acid carbon contains only about 20 per cent of the labelled glucose carbon. The relative rates of incorporation into different cellular fractions indicate that protein synthesis is fastest in the microsomes. In view of the work of the ZAMECNIK group (1954, 1955), and of the findings of the WAELSCH and of the RICHTER groups, this is not surprising.

Since each of the cellular fractions contains several proteins, possibly each with a different turnover rate, small amounts of highly active proteins may account for the radioactivity of the whole fraction, as suggested by LAJTHA *et al.* (1957).

The effect of convulsions, or of stimulation, on the incorporation of ^{14}C into proteins can be divided into three phases. During the first, which comprises the duration of the convulsions, the incorporation rates are increased. According to previously reported experiments (GEIGER *et al.*, 1955), there is also a breakdown and oxidation of the non-carbohydrate material of the brain cells during the convulsive activity. The contradiction implied in these findings is, however, only apparent. Each of the large number of brain cells involved in convulsive activity, after having fired, is able to

VOL.
5
1959/6

resynthesize some of the material during its refractory period, at which time other cells are firing. Thus, the refractory periods of neurons and asynchrony of the neuronal activity during convulsions can account for the increased rate of ^{14}C incorporation into proteins during convulsions.

During the second phase, immediately following convulsive activity, there is a lag in ^{14}C incorporation, during which period apparently no resynthesis of proteins occurs, but rather a loss of ^{14}C is seen. It is difficult to understand how ^{14}C incorporation into proteins should come to a stand-still at a time of reduced physiological activity. If we superimpose onto this picture the rates of oxygen consumption, glucose uptake from the blood, and lactic acid production during convulsions (GEIGER, 1957), and the reduced concentration of ^{14}C in the respiratory CO_2, some correlation among these data can be suspected. According to this picture, the soluble pool of metabolites present in the brain at the beginning of convulsions is rapidly used up during the convulsions by high-rate oxidative processes and glycolysis. Moreover, during this period, the rate of uptake of glucose from the blood is considerably reduced. Thus, at the end of a period of convulsions lasting for 10 to 15 min, the soluble pool of metabolites which was present in the brain at the start is exhausted and possibly the ^{14}C concentration is diluted by the influx of cold carbon from the breakdown of lipids, etc. A temporary lack of specific metabolites could account for the absence of protein synthesis, or even for a loss of an active protein fraction mainly from the microsomes.

During the third phase, in the post-convulsive state, which probably lasts for a long period following the stimulation, the rate of ^{14}C incorporation into proteins (synthesis) is again sharply increased. Compared with the rather low incorporation rates in the resting state, the high rates of incorporation during and after stimulation suggest that a high rate of protein turnover is associated with the activity metabolism of the brain. This idea would also be supported by previous observations (GEIGER, 1957) which show increased breakdown of nitrogen-containing substances (GEIGER *et al.*, 1956) and the prevalent oxidation of non-carbohydrate substrates by the brain during convulsions. Earlier reports on the effects of stimulation on protein turnover in the brain are mostly contradictory. GAITONDE and RICHTER (1956) found no effect beyond a slight lowering of methionine uptake. Similar findings were reported by ROSENGARDT and MASLOVA (1956). VLADIMIROV (1958), on the other hand, found that when physiological activity was inhibited by narcosis or hypothermia or both, the incorporation of radio-active precursors into proteins was drastically reduced. The time factor seems to be of importance for the understanding of previous stimulation experiments. Thus, PALLADIN, BELIK, and KRACHKO (1957) investigated the effects of stimulation on the turnover rate of brain proteins at different time intervals after stimulation was started. Two and a half hours after administration of phenamine (2 hours after methionine subcutaneously), an increased protein turnover was observed. Four hours after injection, there was no difference between stimulated and control animals. Twelve and 24 hr after injection only a tendency toward higher uptake was observed.

The higher rates of protein turnover, indicated by a higher rate of incorporation of radioactive ^{14}C from glucose, induced by stimulation of the brain, suggest the possiblity of tracing the sites of physiological activity in the brain with the use of radioautography.

SUMMARY

1. In brain perfusion experiments *in vivo* and *in situ*, uniformly ^{14}C labelled glucose was added to a simplified perfusion blood of known composition and the incorporation of ^{14}C into the proteins of the brain cortex was followed at rest and during activity.

2. The rates of incorporation of [^{14}C] glucose into the proteins of the brain cortex at rest was considerably slower than into free amino acids.

3. Convulsive activity increased the incorporation rates of ^{14}C into proteins. During the convulsions, the incorporation rate was increased. Immediately after the cessation of the convulsions a lag period of about 15 minutes followed, during which ^{14}C incorporation into proteins entirely ceased and some loss of ^{14}C occurred, mainly from the microsomes. After the lag period, the incorporation rates of ^{14}C again increased 2 to 4-fold over the resting incorporation rates.

4. The incorporation of ^{14}C into various cell fractions was examined. The specific activity of microsomal protein was the highest and that of the mitochondria the lowest. However, most of the ^{14}C incorporated into protein was in the mitochondria as this fraction constitutes about 54 per cent of the total brain proteins.

5. The same characteristic pattern of ^{14}C incorporation which was found during stimulation in the whole cortex was also seen in each cellular fraction.

6. From the microsomes an active fraction was separated.

7. The significance of these finding is discussed.

VOL.
5
1959/6

REFERENCES

BARKULIS S. S., GEIGER A., KAWAKITA Y., AGUILAR V. (1960) *J. Neurochem.* **5,** 339.

BELOFF-CHAIN A., CATANZARO R., CHAIN E. B., MASI I. and PORCHIARI F. (1955) *Proc. Roy. Soc. B*, **144,** 22.

CLOUET D. H. and RICHTER D. (1959) *J. Neurochem.* **3,** 219.

GAITONDE M. K. and RICHTER D. (1955) *Biochem. J.* **59,** 690.

GAITONDE M. K. and RICHTER D. (1956) *Proc. Roy. Soc.* **145,** 83.

GEIGER A. *et al.* (1955) and (1956).

GEIGER A. and MAGNES J. (1947) *Amer. J. Physiol.* **149,** 517.

GEIGER A. (1957). In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 245. Pergamon Press, London.

GEIGER A. (1958) *Physiol. Rev.* **38,** 1.

GEIGER A., DOMNAS A., KAWAKITA Y. and NEBEL L. (1958) *Fed. Proc.* **17,** 52.

GEIGER R. S. (1956) *Fed. Proc.* **15,** 71.

LAJTHA A., FÜRST S., GERSTEIN A. and WAELSCH H. (1957) *J. Neurochem.* **1,** 289.

LITTLEFIELD J. W., KELLER E. B., GROSS J. and LAMECNIK P. C. (1955) *J. biol. Chem.* **217,** 111.

LOWRY O. H., ROBERTS N. R., LEINER K. Y., WU M. L. and FARR A. L. (1954) *J. biol. Chem.* **207,** 1.

PALLADIN A. V. (1958) In *Radioisotopes in Scientific Research*, (Edited by EXTERMAN R. C.) Vol. III, p. 403. Pergamon Press, London.

PALLADIN A. V., BELIK Y. V. and KRACHKO L. L. (1957) *Biochimica* **22,** 359.

PALLADIN A. V. and VERTAIMER N. (1955) *Dokl.-Akad. Nauk SSSR* **102,** 319.

ROBINS E., SMITH D. E., EYDT K. M. (1957) *J. Neurochem.* **1,** 54.

ROSENGARDT V. I. and MASLOVA (1956) Quoted by PALLADIN *et al.* (1957).

SACHS H. (1957) *J. biol. Chem.* **228,** 23.

SCHNEIDER W. E. (1945) *J. biol. Chem.* **161,** 293.

VLADIMIROV G. E. (1958) In *Radioisotopes in Scientific Research* (Edited by EXTERMAN R. C.) Vol. III, p. 432. Pergamon Press, London.

WAELSCH H. (1957) In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 43. Pergamon Press, London.

WAELSCH *et al.* (1958).

ZAMECNIK P. C. and KELLER E. B. (1954) *J. biol. Chem.* **209,** 337.

Journal of Neurochemistry, 1960, Vol. 5, pp. 323–338. Pergamon Press Ltd. Printed in Northern Ireland

MAJOR PATHWAYS OF GLUCOSE UTILIZATION IN THE BRAIN IN BRAIN PERFUSION EXPERIMENTS *IN VIVO* AND *IN SITU**

A. GEIGER, Y. KAWAKITA† and S. S. BARKULIS‡
with the technical assistance of
L. NEBEL, R. STEPHENSON, D. LING and W. SCRUGGS
with a description of the mathematical treatment by
G. H. SANDBERG
Research Laboratories, Department of Psychiatry, University of Illinois College of Medicine

(*Received* 30 *October* 1958)

ALTHOUGH glucose is the only substance which is taken up by the brain in large enough quantities from the blood to satisfy its energy requirements (HIMWICH, 1951), biochemical investigations of recent years have shown a not inconsiderable metabolism of proteins, amino acids, and lipids in the brain (WAELSCH, 1957; ROSSITER, 1957; VLADIMIROV, 1958; PALLADIN, 1958). It has been shown also that a number of physiological functions and the respiration of the brain can be maintained in the complete absence of glucose in brain perfusion experiments, with the blood circulation of the brain separated from the rest of the body of the animal, for up to two hours (GEIGER, MAGNES and GEIGER, 1952). Similar experimental evidence also indicated that during convulsions the increased rates of oxidation and brain activity are supported mainly by endogenous non-carbohydrate substances (GEIGER, MAGNES and DOBKIN, 1954).

The fact that glucose is the only substrate taken up from the blood into the brain in large enough quantities to sustain its energy requirements suggests glucose as the carbon source for all the main metabolic processes in the brain including the production, by exchange or by synthesis, of most amino acids, proteins, lipids, etc. Thus, in brain perfusion experiments and in the presence of ^{14}C-labelled glucose the ^{14}C-labelling of brain components can be expected to indicate which of its chemical components are produced by the brain and the rates of their production. On the other hand, after the completed exchange of the pre-existing brain glucose and of its intermediates with the ^{14}C-labelled blood glucose, the relative proportions of ^{14}C and cold carbon in the respiratory CO_2 can show the extent to which non-glucose carbon is utilized in the respiratory metabolism. It was hoped that in this first part of the work the relative proportions in which glucose and endogenous substances other than glucose participate in the production of respiratory CO_2 in the brain could be obtained from analyses of the respiratory CO_2 and from other related data. An attempt was also

* This work was supported by a grant from the National Institute of Neurological Diseases and Blindness, B-413.
Preliminary reports of the present findings have been presented (GEIGER *et al.*, 1958).
† Present address: Department of Neuropsychiatry, Osaka City University Medical School, Osaka, Japan.
‡ Present address: Research Department, Ciba Pharmaceutical Products, Inc. Summit, New Jersey.

made to detect eventual changes in metabolic patterns under diverse physiological conditions by the proportion of ^{14}C and cold carbon in the CO_2 produced.

In these experiments, as ^{14}C-labelled glucose was mixed into the perfusion blood, ^{14}C appeared in the respiratory CO_2 within a minute, and its concentration rose steadily until it attained a constant level within about 30 min. This relationship then remained constant for the duration of the experiments, usually 30 to 60 min. The first phase of rising ^{14}C concentration in the CO_2 is taken to indicate the exchange of pre-existing glucose and its intermediates with the labelled glucose supplied by the blood. It is assumed that at the end of this stage the exchange with the direct metabolites and other quickly-exchangeable compounds has been completed.

The second phase, namely, the period during which the ^{14}C concentration in the CO_2, attained at the end of the rising phase, remained constant, reflects the entry of non-radioactive carbon into the pathways of carbon dioxide formation at a steady rate, maintaining a constant ratio with radio-carbon from the entering [^{14}C] glucose. This constancy is, of course, only relative, reflecting the entry of cold carbon from comparatively very large pools (proteins, lipids, nucleic acids, and possibly acid-soluble compounds) which, by virtue of their size, acquire an insignificant specific activity within the course of the perfusion experiments. This ratio is considered to indicate the proportion in which glucose and other non-glucose compounds are participating in respiratory CO_2-formation. The source of this non-radioactive carbon is at present unknown.

The experiments to be reported here indicate that, in addition to glucose, comparatively large amounts of other substances are being constantly oxidized to CO_2, and that ^{14}C derived from glucose is being incorporated into amino acids and other compounds in the brain in the resting state.

During increased metabolic activity as produced by metrazol convulsions the increase in the rate of oxidation is supported mainly, if not exclusively, from non-carbohydrate sources.

EXPERIMENTAL

The brain perfusion method as previously described (GEIGER and MAGNES, 1947; GEIGER, MAGNES and DOBKIN, 1954; GEIGER, 1958) was used with minor modifications. The criteria of the viability of the preparation were those described previously (GEIGER, 1958). From 700 to 900 ml of the 'simplified blood' containing 60–90 mg of carrier glucose per 100 ml were circulated through the brain at an average blood pressure of 80–90 mm Hg to maintain in the resting state a blood-flow rate of 120–160 ml/min per 100 g brain. Cerebral oxygen consumption and blood-flow rate were continuously recorded. Uniformly ^{14}C-labelled glucose was added to the perfusion blood, usually 20–30 min after the start of the perfusion, when the oxygen consumption of the brain had reached a steady state, the ECG pattern was normal, and the responses of the brain to stimuli (corneal reflex, pupillary reflex, ECG reaction to touch of limbs and skin) were good. After the addition of 70–130 μC of [^{14}C] glucose to the blood reservoir (the uniformly-labelled glucose used [Nuclear Chicago] had about 2–3 μC per mg), 20–30 min were allowed for proper mixing of the [^{14}C] glucose with the blood and for the equilibration of the glucose originally present in the brain with the glucose in the blood, before taking brain cortex samples. Complete equilibration of the brain glucose with the blood glucose occurred, usually within less than 10 min as seen in a number of previous experiments not included in this paper. Arterial and venous samples for various analyses were taken simultaneously at various time intervals. In the earlier experiments, while taking the blood samples, the whole venous outflow was diverted for a period of 10 min, after passing the oxymeter cells, into a previously collapsed, thin-walled plastic bag which was kept on ice. Portions of these samples were taken under mercury for analysis. The arterial sample was taken at the same time from the overflow of the pressure regulator. For CO_2 analysis, 15 ml portions were distributed into test tubes under liquid paraffin

VOL.
5
1959/6

in an ice bath. Other portions were treated with perchloric acid (PCA) for the analysis of acid-soluble substances.

Owing to the rather high blood-flow rates, the differences in glucose concentration between arterial and venous blood were small. Therefore, these measurements were repeated on blood samples taken from the circulating blood in the reservoir at longer time intervals with accurate check on the volume of the circulating blood in the reservoir and apparatus and correction for the glycolysis of the blood itself. The rates of glucose uptake for the data in this paper were measured with both methods. In the more recent experiments (1959), the blood samples for CO_2 analyses were collected in cut-down 10 ml volumetric pipettes, which were inserted as by-passes into the arterial and venous circulation of the apparatus and contained circulating blood all the time. At the time of the collection of blood samples the rubber tubes connecting these pipettes with the apparatus were clamped, and the blood samples were transferred under mercury. Care was taken not to change the arterial and venous blood pressures during the collection of blood samples. Five minutes after the addition of [^{14}C] glucose to the blood reservoir, when mixing was complete, blood samples were treated with PCA. In these acid filtrates the blood-glucose concentration was determined by the FOLIN–MALMROS (1929) method before and after treating the neutralized filtrates with glucose oxidase, and their radioactivity was counted with a thin-window gas flow counter. In those experiments in which the glucose was not isolated as osazone, the specific activity of the blood-glucose could be calculated from these analyses and counts, as the only radioactive compound in the blood in measurable amounts at this time was glucose. The accuracy of this procedure was ascertained in a number of experiments in which the glucosazones were prepared by the method of FELLER, STRISOVER and CHAIKOFF (1950). The specific activities for glucose obtained from the PCA filtrates were practically identical with those found in the glucosazones. CO_2-analyses were completed on the day of the experiments. The total CO_2 content of the blood was determined on three 1 ml portions by the manometric VAN SLYKE method. The radioactivity of the CO_2 was determined as in the earlier experiments (1957–58) by precipitation of the CO_2 as $BaCO_3$. For these analyses, the CO_2 was expelled from two 10 ml portions of the blood by acidification and aeration and collected quantitatively in CO_2-free N-KOH. Portions of the KOH solution were precipitated as $BaCO_3$. After the usual washing and drying, the $BaCO_3$ was transferred quantitatively onto stainless steel planchettes, dried, and counted.

In the majority of the experiments reported (GEIGER, 1959), however, the ^{14}C in the respiratory CO_2 was determined by a different method which proved to be much more exact and also much easier to handle than the $BaCO_3$ precipitation. In these analyses, 4 ml each of the arterial and venous blood samples were transferred under mercury from the 10 ml volumetric pipettes in which they had been collected into test tubes under 1 ml of 0·5 N-NaOH to bind the free CO_2. One ml of the NaOH–blood mixture was transferred into a previously prepared Conway dish. Into the mid-section of each Conway dish a small glass cup, 15 mm in diameter and 8 mm high, was placed. These cups were previously treated with silicone solution to avoid the creeping over of the 0·3 ml of about 0·5 M 'Hyamine' solution in methanol which was measured into it. The Hyamine-hydroxide solutions were made according to PASSMANN, RADIN and COOPER (1956) with EISENBERG's modification (1958). The 'Hyamine' solution serves the double purpose, first to absorb the CO_2 in the Conway dish, and later to keep it in solution in the scintillation mixture. 1 ml of the blood mixture with NaOH was placed in one side of the outer compartment of the Conway dish and 1 ml of N-lactic acid was placed

TABLE 1.—ACCURACY OF DETERMINATION OF RADIOACTIVITY IN CO_2 OF BLOOD BY THE METHOD DESCRIBED IN THE TEXT

The volume was 0·8 ml of blood for each determination.

Sample	CO_2 in sample	Counts/min
Sample I arterial blood	0·325	323 325 321
Sample II venous blood	0·354	1724 1724 1724

into the other side. After closing the dish with a vaseline-covered glass plate, the blood and lactic acid were mixed by gently agitating it in a circular movement on a flat surafce. After 90 min at room temperature (about 24°), the diffusion of the CO_2 into the 'Hyamine' solution was complete. The Conway dish was opened, and the glass cup in the central well containing the Hyamine-hydroxide was picked up by forceps and dropped into a counting vial of the scintillation counter. In order to keep the Hyamine in solution and to replace the methanol that evaporated, 1 ml of methanol was added, followed by 10 ml of scintillator solution. This contained 4 gm of 1:4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) and 100 mg of 2:5-diphenyloxazol (PPO) in 1 l. of toluene. After thorough mixing and cooling, the vials were counted on a 'Packard tri-carb liquid scintillation spectrometer.' These determinations were made first in triplicate, and later in duplicate. The agreement between duplicate determinations was excellent, and the results were very accurate (Table 1).

Other portions of the arterial and venous blood were precipitated with PCA (final concentration, 7%) for the determination of glucose and of other acid-soluble components. Portions of the PCA filtrates were also neutralized with NaOH, cooled, and, after removal of the precipitate, plated, dried, weighed, and counted.

Brain cortex samples were excised at stated intervals, quickly frozen over dry ice, and analysed as described in the following paper.

At the end of the experiments, the whole brain was quickly taken out, washed in ice-cold Ringer's solution, and after removal of the excess Ringer's solution quickly frozen on dry ice. One-half of this brain was homogenized later for 'total brain' analyses. The acid-extracts were plated and counted. Lactic acid was determined in most blood and brain samples by the BARKER and SUMMERSON (1941) method.

Amino acids and their radioactivity were determined in the PCA extract of the blood and of the brain in the earlier experiments by elution from a Dowex 50 × 4 column with 6 N-HCl.

In the later experiments, an extensive study was made simultaneously with the present studies on the ^{14}C incorporation from glucose into the amino acids in the brain by BARKULIS *et al.* (1960).

The experimental conditions and the blood composition were kept constant throughout each experiment.

After proper mixing of the labelled glucose with the blood, the specific activity of the glucose in the perfusion blood remained constant throughout the experiment, as no new formation of glucose occurred. This was ascertained in several experiments. Of the 400–600 mg of glucose present in 500–800 ml of perfusion blood at the start of the experiments, usually about 100 mg were utilized in 1 hr by the brain at a steady rate.

The blood flow rate and the arterio-venous oxygen differences were measured directly and continuously with the flowmeter and the oximeter as described previously.

At the end of each experiment, the whole brain, including the medulla, to the level of the venous clips, was excised, frozen, and weighed.

CALCULATIONS

The blood flow rate and the oxygen consumption were calculated for 100 g brain and for a 1 min period.

The glucose taken up from the blood and the lactic acid produced by the brain were also calculated for 100 gm brain and for a 1 min period in milligrams by multiplying the differences of glucose concentrations (mg/ml) between arterial and venous blood (as obtained by analysis) by the blood flow rate. In addition, the glucose uptake was also calculated from the differences of the ^{14}C-counts in the PCA-extracts of the arterial and the venous blood. Owing to the relatively high blood flow rate, the glucose uptake and lactic acid output were also calculated from analyses made on blood samples taken from the reservoir at 15-min intervals. An accurate account of the blood volume was kept.

The total amount of carbon dioxide produced by 100 gm brain in 1 min was calculated similarly by using the arteriovenous CO_2 differences determined by manometric VAN SLYKE analyses in the blood. Since we found, in a very large number of earlier, similar experiments, the R.Q. of the 'resting' brain to be at, or very near, 0·95, we calculated the CO_2 output in some experiments from this R.Q., and from the measured oxygen consumption.

In this and the following paper, glucose, lactic acid, CO_2, and amino acids are expressed as mg carbon. This was calculated by multiplying each mg of glucose or lactic acid by 0·4, each ml of CO_2

VOL. 5 1959/6

by 0·536, and each mM of amino acid by 54. We arrived at this figure for amino acid carbon by averaging the 4- and 5-carbon amino acids found in the brain.

The equation for the calculation of the $^{14}CO_2$ production by the brain was formulated by G. H. SANDBERG, and its derivation is described in the Appendix. This formula takes into consideration the rate of increase of $^{14}CO_2$ in the arterial blood at the time of collection of blood. Owing to the fact that $^{14}CO_2$ is constantly fed into the blood by the brain, the concentration of $^{14}CO_2$ in the arterial blood is increasing. On the other hand, the oxygenator of the apparatus constantly extracts $^{14}CO_2$ from the blood, thereby reducing this increment.

According to this equation:

$$R_m = R_v - R_a + \frac{v}{R_v}\frac{d}{dt}(R_a)$$

in this, R_m = metabolic production of $^{14}CO_2$
R_a = flow rate of $^{14}CO_2$ ($c_a \times K_a$)
c_a = specific activity of CO_2 in arterial blood in counts/min per ml CO_2 at a given time
k_a = input rate of CO_2 from arterial blood into brain
R_v = flow rate of $^{14}CO_2$ ($c_v \times K_v$)
c_v = specific activity of CO_2 in venous blood in counts/min per ml CO_2 at a given time
K_v = output rate of CO_2 from brain into venous blood
v = ml CO_2 in 100 g brain*

From the amounts of $^{14}CO_2$ produced by the brain, as calculated by this equation, the amounts of glucose-carbon present in the respiratory CO_2 (in mg) were calculated by dividing the counts by the specific activity of the glucose-carbon in brain and blood. Eventual variations of v, which, under the present experimental conditions are small, would result in only negligible change of R_m, since the numerical values of this calculation did not exceed 5% of R_m.

RESULTS AND DISCUSSION

It was desirable to ascertain in a preliminary study the time factors of complete exchange of CO_2 and of glucose between brain and blood. It is known that blood-CO_2 exchanges very quickly with that of the tissues. For these experiments the technique was slightly modified. Blood of the same composition as that in the rest of the perfusion apparatus was mixed with a small amount of ^{14}C-labelled $NaHCO_3$ in a separatory funnel which was suspended at such a height as to give a hydrostatic pressure equivalent to the blood pressure in the perfusion apparatus. This funnel was connected by a thin rubber tube, through the heating coil of the arterial side, to the brain of the cat. Before perfusing this ^{14}C-labelled bicarbonate through the brain, a normal perfusion was started during which the oxygen consumption, blood flow rate, etc., of the brain were measured. At a given time, the blood supply of the brain was switched over to the ^{14}C-containing reservoir and the venous blood was diverted into collecting tubes. The blood was collected every minute in a separate container. Analyses of CO_2 and counts of the $^{14}CO_2$ were made as usual on the blood in the reservoir and on the venous blood.

After the second minute, the radioactivity of the venous blood did not change during 6 consecutive 1 min-periods (Fig. 1); this result indicates that during less than 2 minutes complete exchange of the brain-CO_2 had taken place. These 2 minutes include the time (50 sec) which was necessary for the passage of the blood through the tubes to reach the brain and collecting vessels. The difference between the specific activities of the arterial and venous blood represents the diluting effect of the CO_2

* The CO_2 content of the brain, determined in a number of experiments by homogenizing freshly-excised pieces of brain in a small volume of N-KOH in a glass homogenizer with care to avoid as well as possible loss or absorption of CO_2, very nearly equaled that of the arterial blood. Therefore, in many experiments, v was taken to equal the CO_2 concentration of arterial blood.

produced by the brain, which under these conditions is not radioactive. This experiment indicated that the rate of exchange of CO_2 in blood and brain is rapid enough for the measurements which were made in the experiments below.

The exchange of glucose between blood and brain is necessarily a somewhat slower process. Even so, it should be completed within a comparatively short time.

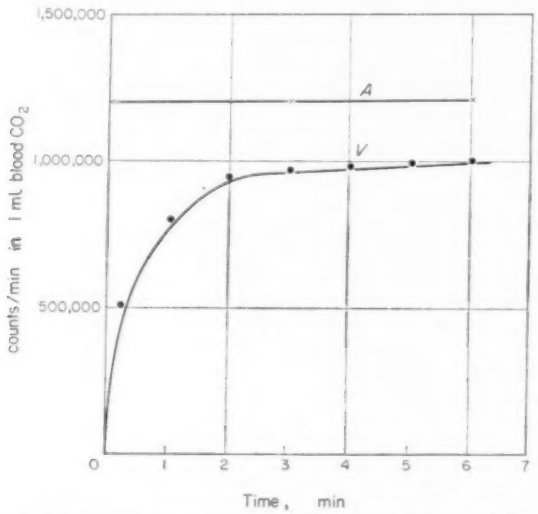


FIG. 1.—Rate of exchange between the CO_2 of brain and blood. Line A shows the specific activity of the arterial-CO_2; curve V that of the venous blood-CO_2. The time required for the blood to reach the brain through the rubber tubing of the heating system was 50 sec.

The true glucose content of the brain as determined with glucose oxidase at the time when ^{14}C-labelled glucose is added to the blood is usually about 40 mg per 100 g. However, the rate at which glucose is utilized by the brain is about 7–8 mg glucose per 100 g brain per min. Thus, the glucose originally present in the brain should be used up within about 5 min. In addition, under our experimental conditions the blood flowing through the brain carries about 120 mg of ^{14}C-labelled glucose through each 100 g brain every minute. Thus, utilization and exchange should reduce the time necessary for complete exchange to less than 5 min. This has been verified by comparing the glucosazones prepared from the blood and from PCA extracts of brain cortex pieces excised at different time intervals. In one experiment (March 3), the specific activity of glucose in the blood was 138,000 counts/min per mg, and that of the brain showed 124,000 and 141,000 counts/min per mg glucose in two determinations after 20 min of perfusion. In another experiment (April 30), after 28 min of perfusion, 142,000 counts/min were found in 1 mg of blood glucose, and 128,000 and 146,000 counts/min per mg in the brain glucose.

PCA extracts of brain cortex, excised about 20 min after the addition of ^{14}C-labelled glucose, contained much more radioactivity than could be accounted for by the glucose present in the brain (BARKULIS *et al.*, 1960). The counts in excess of those of glucose were due to other acid-soluble components, which were formed in the brain from glucose (such as lactic acid, amino acids, and other organic compounds).

VOL.
5
1959/6

BELOFF-CHAIN *et al.* (1955) first showed the synthesis of amino acids from added ^{14}C-labelled glucose by brain slices.

The relationship between the amounts of oxygen consumed by the brain and the amount of glucose made available for oxidative metabolism by uptake from the blood had to be re-examined under our experimental conditions in order to determine whether or not a steady-state relationship between glucose uptake and utilization is maintained during perfusion.

The rate of glucose uptake was measured by glucose- and lactic acid-analyses in simultaneously taken arterial and venous blood samples, and also in blood samples taken from the reservoir at 15-min intervals. The data (Table 2) indicate the

TABLE 2.—RATES OF GLUCOSE UPTAKE, LACTIC ACID PRODUCTION, AND CO_2 PRODUCTION AS DETERMINED BY ANALYSIS OF THE BLOOD IN THE APPARATUS AT DIFFERENT TIME INTERVALS DURING PERFUSION WITH UNIFORMLY ^{14}C-LABELLED GLUCOSE

		Calculated as mg carbon/100 g brain per min		
Expt.	Perfusion time with ^{14}C-labelled glucose (min)	Glucose* uptake	Lactic acid output	Respiratory† CO_2
June 10, 1959	0–35	5·71	2·46	2·69
May 12, 1959	20–50	4·84	1·88	2·54
April 30, 1959	20–68	3·24	0·584	2·62
June 4, 1959	36–66	3·58	1·45	2·3
June 24, 1959	16–28	4·97	2·34	2·73

* The standard error in these determinations is $\pm 8\%$ of the figures presented.
† The standard error in these determinations is $\pm 4\%$ of the figures presented.

amounts of glucose taken up which were available for CO_2 production after subtracting the lactic acid released into the blood. The glucose uptake was measured also from differences in counts in the acid extracts of the blood (Table 3). Correction for lactic acid output is not necessary in the latter Table. The amount of glucose taken up by the brain, after subtracting from it the lactic acid released, is sufficient to account for the CO_2 produced by the brain, usually within 85 to 100 per cent (Tables 2 and 3). The amount of lactic acid produced, which accumulates in the brain, is relatively small, averaging 0·35 mg per 100 g brain per min.

After a perfusion period of 90–100 min, the glucose uptake often does not keep pace with the oxygen consumption. After these perfusion times, in spite of undiminished rates of CO_2 production, the amounts of glucose taken up become progressively smaller; this result indicates the oxidation of endogenous substances which are now not replaced by or resynthesized from glucose. For this reason, all our experiments were kept within the period of equivalency between glucose uptake and CO_2 production (Table 7).

In 11 experiments, the relative proportion of ^{14}C derived from glucose was measured in the respiratory CO_2 in the 'resting' state (Table 4), as affected by 'Metrazol' convulsions (Table 5), and as affected by narcosis (Table 6). All these data show that

TABLE 3.—GLUCOSE UPTAKE AND UTILIZATION BY THE BRAIN DETERMINED BY COUNTING THE ACID EXTRACTS OF BLOOD IN THE APPARATUS AT DIFFERENT TIME INTERVALS DURING PERFUSION WITH UNIFORMLY ^{14}C-LABELLED GLUCOSE

Expt.	Perfusion time with ^{14}C-glucose (min)	Specific activity of glucose (counts/min per mg carbon $\times 10^{-3}$)	Calculated for 100 g brain/min		
			Uptake (counts $\times 10^{-3}$)	Uptake (mg C)	Output in respiratory CO_2 (mg C)
June 4, 1959	36–66	246	531	2·16	2·30
Apr. 30, 1959	20–68	354	920	2·60	2·62
May 12, 1959	20–50	262	770	2·94	2·54
June 10, 1959	22–46	328	1,042	3·22	2·45
May 17, 1958	24–30	202	484	2·40	2·36
Feb. 6, 1958	30–35	122·5	299	2·74	2·30
May 28, 1958	20–30	179	409	2·28	2·44
Mar. 12, 1958	42–52	344	1,265	3·68	4·10
Apr. 7, 1959*	26–52	410	1,750	4·27	3·68
Mar. 3, 1959*	20–54	345	1,152	3·34	3·53
Apr. 3, 1958*	24–36	125	380	3·04	3·28

* Includes part of metrazol convulsions

TABLE 4.—CARBON^{14} DERIVED FROM GLUCOSE IN THE RESPIRATORY CO_2 IN THE 'RESTING' STATE DURING PERFUSION WITH UNIFORMLY ^{14}C-LABELLED GLUCOSE

Expt.	Perfusion time with [^{14}C]-glucose (min)	Specific activity of glucose C (counts/min per mg C $\times 10^{-3}$)	Calculated for 100 g brain/min				
			Blood flow rate (ml)	Carbon in CO_2 produced (mg)	Counts in CO_2		
					Counts/min $\times 10^{-3}$	Counts in CO_2 expressed as mg C derived from glucose	Carbon in CO_2 derived from glucose (%)
Apr. 30, 1959	18	354	166	2·62	168·0	0·485	18·5
	34		167	2·55	235·5	0·665	26·0
	48		154	2·46	257·0	0·725	29·5
	66		156	2·47	256·0	0·722	29·2
May 12, 1959	20	262	102	2·60	116·0	0·443	17·1
	34		165	2·70	165·0	0·630	23·3
	50		161	2·58	184·0	0·703	27·3
	64		150	2·28	175·0	0·668	29·3
June 10, 1959	3	328	160	2·39	28·2	0·089	3·72
	8		154	2·34	87·9	0·268	11·5
	22		152	2·25	214.0	0·654	27·9
	37		178	2·20	230·0	0·704	27·6

VOL. 5 1959/6

TABLE 5.—CARBON[14] DERIVED FROM GLUCOSE IN THE RESPIRATORY CO_2, AT REST AND DURING 'METRAZOL' CONVULSIONS

Expt.	Perfusion time with [^{14}C]-glucose (min)	Specific activity of glucose C (counts/min per mg C $\times 10^{-3}$)	Calculated for 100 g brain/min				
			Blood flow rate (ml)	Carbon in CO_2 produced (mg)	Counts in CO_2		
					Counts/min $\times 10^{-3}$	Counts in CO_2 expressed as mg C derived from glucose	Carbon in CO_2 derived from glucose (%)
March 3, 1959	18	345	210	2·12	—	—	—
	32		175	2·45	256·5	0·743	30·3
	40		METRAZOL				
	46		215	5·10	105·0	0·305	6·0
	54		250	4·74	—	—	—
April 7, 1959	14	410	139	2·48	158·6	0·387	15·6
	28		128	2·50	328·6	0·803	32·1
	38		METRAZOL				
	40		125	3·84	200·0	0·488	12·7
	54		110	1·97	155·0	0·378	19·2
June 24, 1959	2	237·5	180	2·28	8·1	0·034	1·5
	8		170	2·59	108·0	0·455	17·6
	13		155	2·33	133·5	0·563	24·2
	28		145	2·70	212·0	0·889	32·9
	38		METRAZOL				
	40		192	4·17	262·5	1·105	26·5
	54		168	3·26	245·0	1·030	31·6
February 6, 1958	30	122·5	114	2·44	78·5	0·640	26·2
	65		110	2·35	82·4	0·672	28·6
	76		METRAZOL				
	81		241	4·75	164·2	1·340	28·2
	100		180	3·76	52·0	0·424	11·3
April 3, 1958	36	125	125	2·66	33·2	0·66	24·8
	68		METRAZOL				
	70		140	3·72	71·5	0·572	15·4
	96		155	2·54	31·7	0·620	24·4

the concentration of ^{14}C in the CO_2 steadily increases during the first 25–30 min after the addition of ^{14}C to the perfusion blood until it reaches a concentration of 29–32 per cent. From this point on, the concentration of ^{14}C in the respiratory CO_2 remains constant (Fig. 2). The first ascending phase of the ^{14}C concentration in the respiratory CO_2 indicates the exchange of glucose between the brain and the blood and the exchange of the preformed intermediary products with those newly formed from ^{14}C

TABLE 6.—EFFECT OF 'NEMBUTAL' NARCOSIS ON THE AMOUNTS OF ^{14}C DERIVED FROM GLUCOSE IN THE RESPIRATORY CO_2

Expt.	Perfusion time with [^{14}C]-glucose (min)	Specific activity of glucose C (counts/min per mg C $\times 10^{-3}$)	Calculated for 100 g brain/min				
			Blood flow rate (ml)	Carbon: in CO_2 produced (mg)	Counts in CO_2		
					Counts/min $\times 10^{-3}$	Counts in CO_2 expressed as mg C derived from glucose	Carbon in CO_2 derived from glucose (%)
April 23, 1959	16	252	200	2·30	92·1	0·370	16·0
	30		197	2·55	176·8	0·705	27·5
	42		Nembutal added to perfusion blood				
	46		180	1·89	167·0	0·664	35·0
	60		177	1·55	136·0	0·540	35·0
May 5, 1959	22	300	155	2·45	161·0	0·537	21·9
	36		153	2·33	188·5	0·630	27·0
	50		Nembutal added to perfusion blood				
	56		185	1·85	288·2	0·965	52·1
	76		150	1·78	272·0	0·906	51·0
June 4, 1959	22	246	162	2·75	130·0	0·529	19·2
	36		164	2·38	165·5	0·673	28·3
	46		Nembutal added to perfusion blood				
	52		178	1·68	141·0	0·573	34·0
	66		165	1·28	94·2	0·383	29·9

TABLE 7.—TOTAL ^{14}C TAKEN UP (AS GLUCOSE BY 1 g BRAIN) DURING 1 hr PERFUSION WITH UNIFORMLY ^{14}C-LABELLED GLUCOSE AND THE ^{14}C FOUND IN THE BRAIN AND ELIMINATED IN THE RESPIRATORY CO_2 DURING THE SAME PERIOD OF TIME

Expt.	Perfusion time with ^{14}C-labelled glucose (min)	Calculated for 1 g brain and 1 hr perfusion time			
		^{14}C in acid soluble fraction (mg)	^{14}C in respiratory CO_2 (mg)	Sum of ^{14}C in acid soluble fraction and CO_2 (mg)	C in respiratory CO_2 (mg)
June 4, 1959	0–58	0·837	0·290	1·127	1·341
April 23, 1959	0–62	0·968	0·311	1·279	1·214
May 5, 1959	0–58	0·911	0·312	1·223	1·282
May 19, 1959	0–48	0·730	0·208	0·938	1·012
June 10, 1959	0–56	0·874	0·322	1·196	1·385
April 30, 1959	0–56	0·994	0·329	1·323	1·509
Mean		0·886	0·295	1·181	1·291

The figures in this table were obtained by direct counting of the respective fractions, and calculating for 1 g brain and 1 hr perfusion time.

VOL. 5 1959/6

labelled glucose. From this point on, when a constancy in the ratio between ^{14}C and cold carbon has been reached and steadily maintained at the same level for the remainder of the experiment, this ratio indicates the relative proportion at which cold carbon from non-carbohydrate substances is mixed with the ^{14}C of the glucose derivatives to form the respiratory CO_2. The findings presented here confirm those of ALLWEISS and MAGNES (1956; 1958*a*) which were also obtained in perfusion experiments.

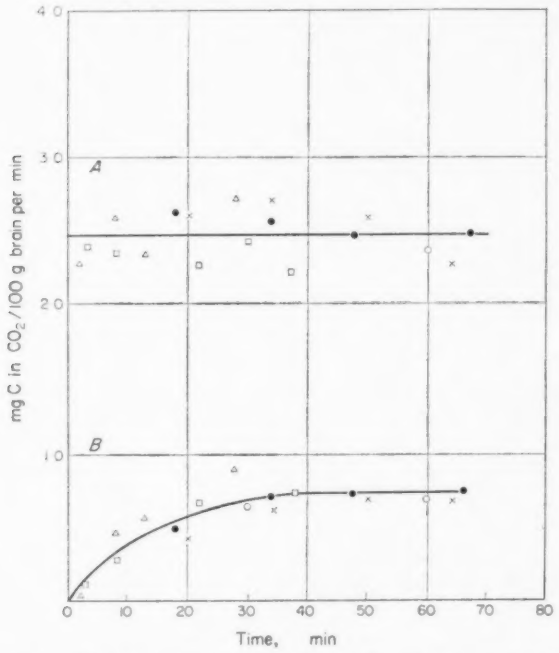


FIG. 2.—The upper line A shows the total carbon in the respiratory CO_2; lower curve B shows the amounts of ^{14}C derived from glucose appearing in the respiratory CO_2 at different perfusion times. Carbon expressed in mg.
a = Apr. 30, 1959; b = May 12, 1959; c = June 10, 1959; d = June 24, 1959; e = Feb. 6, 1958
Key. ● = a × = b □ = c △ = d ○ = e

The glucose exchange should be completed in about 5 min. The concentration of intermediary substances of glycolysis is not very high in the brain, amounting to about 32 mg carbon in 100 g of brain (ABOOD *et al.*, 1952), and that of members of the tricarboxylic cycle is lower. Thus, calculations show that the carbon of the preexisting glycolytic intermediates should have 90 per cent of the specific activity of the incoming glucose carbon within about 25 min. Another large pool, however, which dilutes the precursors of the respiratory CO_2 is that of the free amino acids in which the ^{14}C concentration rose in most experiments to a plateau reached later and at a lower level than in the CO_2 (cf. Fig. 1, BARKULIS *et al.*, 1960).

The fact that the constant ^{14}C concentration which is attained in the CO_2 and amino acids is considerably lower than would be expected for the time required by the level of radioactivity in the glycolytic intermediates after 25 min indicates that unlabelled carbon is being added at some point and at a considerable rate.

The fate of the glucose that has been taken up by the brain in these perfusion experiments and not used for CO_2 production is not quite clear. Most of it is found in the acid soluble fraction (amino acids, lactic acid, and other substances which have not been analysed in these experiments) and in the lactic acid released into the blood, and a small part is in proteins, nucleic acids, and lipids. It is possible that in the later stages of the perfusion experiments, when the rate of glucose uptake is smaller than that of the CO_2 production, more carbon originates from breakdown products of

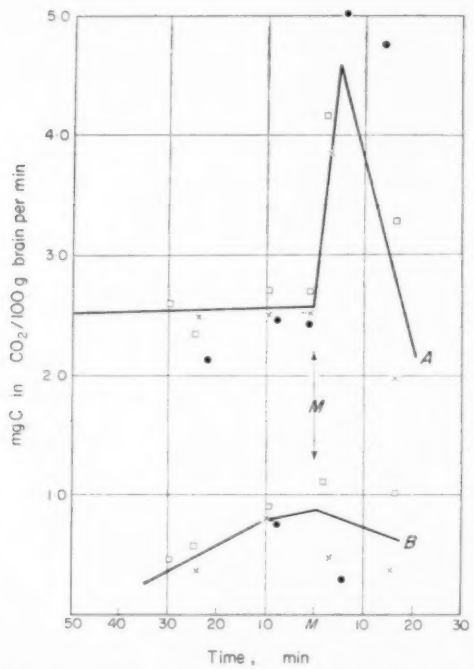


FIG. 3.—Effect of 'Metrazol' convulsions on the ratio between ^{14}C and total carbon in the respiratory CO_2. Upper curve A shows the total amount of carbon in the respiratory CO_2. The lower line B shows the amount of ^{14}C carbon in the respiratory CO_2.
a = March 3, 1959; b = Apr. 7, 1959; c = June 24, 1959.
Key. ● = a × = b □ = c

non-carbohydrate compounds than was the case previously. Under these conditions it is likely that normal resynthesis of these compounds does not occur, and that such changes may occur gradually during the perfusion experiments, thus shifting the equilibrium between the utilization of glucose and of non-carbohydrate compounds in forming CO_2. Work is now in progress to clarify these points.

In all experiments with Metrazol (Fig. 3), it was administered after at least 30 min of perfusion with ^{14}C-labelled glucose to allow enough time for the exchange reactions to be completed. During the Metrazol convulsions, the oxygen consumption and CO_2 output of the brain increased considerably, as was to be expected. However, the total ^{14}C output in the respiratory CO_2 did not increase at all, and in some cases even diminished. The relative ratio between total C and ^{14}C derived from glucose shows that much less carbon is derived from glucose than in the 'resting' state. This

VOL.
5
1959/6

fact clearly indicates that the extra oxygen consumed during the convulsions can be accounted for only by the oxidation of non-glucose or non-carbohydrate substances. This metabolic change has been suggested before (GEIGER, 1956), and has also been shown by ALLWEISS and MAGNES (1958*b*).

With the decreased rate of CO_2 production in nembutal narcosis (Table 6) the ratio of ^{14}C to total C in respiratory CO_2 was somewhat increased; this result indicates a decreased participation of non-carbohydrates in the production of CO_2. In view of the rather small number of experiments made in narcosis, this finding is considered to be preliminary.

Some possible interpretations of the results of this work are discussed in the following paper (BARKULIS *et al.*, 1960) on the basis of data presented there.

SUMMARY

The blood circulation of brains of cats was isolated, with maintenance of the nervous connections with the rest of the body, and the brains were perfused with a 'simplified blood,' containing uniformly ^{14}C-labelled glucose. The main purpose of these experiments was to obtain data on the relative proportions in which glucose and endogenous substances other than glucose participate in the production of respiratory CO_2 in the brain in the 'resting' state and under diverse physiological conditions. This information was obtained by analysing the proportion of ^{14}C and cold carbon in the CO_2 produced, and from other data relative to glucose uptake and utilization.

A modification of existing methods for the accurate counting of the radioactivity in ^{14}C-labelled CO_2 contained in less than 1 ml of blood is described.

The time required for the complete exchange of the total CO_2 of the brain with that of the circulating blood is described. The time required for the exchange of the brain-glucose with that of the blood was measured.

The rate of glucose uptake by the brain equalled the amounts required for the production of the respiratory CO_2 and lactic acid.

Under 'resting' conditions, the ^{14}C concentration in the respiratory CO_2 steadily increased during the first 30 min after the addition of ^{14}C until it reached a concentration of 29–32 per cent. From this point on, the concentration of ^{14}C in the respiratory CO_2 remained constant at this level for the remainder of the experiment. These results are interpreted to indicate that both glucose carbon and cold carbon from endogenous non-carbohydrate contribute to form respiratory CO_2.

During periods of increased rates of CO_2 output, induced by Metrazol convulsions, the total ^{14}C output did not increase over the resting level and in some cases even diminished; this result indicates that the increase in the rate of oxidation during convulsions is supported mainly by the oxidation of non-carbohydrate substances.

During narcosis and reduced CO_2 production there was some increase in the concentration of ^{14}C in the respiratory CO_2.

For the discussion of the interpretation of these results, reference is made to the second paper of this series (BARKULIS *et al.*, 1960).

APPENDIX

We consider the CO_2 pool in the brain during steady-state metabolism, so that the effective amount of CO_2 present, v, is constant and the input flow rates, k_a from arterial blood and k_m from metabolic processes, do not change with time. The

specific activity of CO_2 in the pool and the venous blood, c, depends on the specific activity, c_m, of metabolic CO_2, the specific activity, c_a, of arterial CO_2, and time, t, according to the differential equation for continuity of flow:

$$\frac{dc}{dt} = \frac{k_a}{v} c_a + \frac{k_m}{v} c_m - \frac{(k_a + k_m)}{v} c \tag{1}$$

This equation has a general solution:

$$c = c_0 e^{-\lambda t} + \frac{k_a}{v} \int_0^t c_a(t_1)\, e^{-\lambda(t-t_1)}\, dt_1 + \frac{k_m}{v} \int_0^t c_m(t_2)\, e^{-\lambda(t-t_2)}\, dt_2 \tag{2}$$

The exponential factors contain the time constant, $\lambda = \frac{k_a + k_m}{v}$ characteristic of mixing in the CO_2 pool. The specific activity of the metabolic CO_2 is in general a function of time, $c_m(t)$, which depends on the experimental procedure; in the experiments analysed, this function changes slowly compared with the exponential function for mixing in the CO_2 pool. For the purpose of this analysis which takes account of the mixing process it can be assumed constant within the accuracy of the measurements. The specific activity of the arterial CO_2 is also a function of time, $c_a(t)$, which depends on the accumulation of active CO_2 in the brain and venous blood and on the characteristics of the aerator through which it is returned to the arterial circulation. We use a linear approximation,

$$c_a(t) = c_a(t_1) + r_a \,.\, (t - t_1),$$

for $t > 0$, where c_a and r_a can be evaluated from the experimental measurements.

With the above substitutions, we can carry out the integrations indicated in (2) to get:

$$c = \frac{k_m}{k_a + k_m} c_m(1 - e^{-\lambda t}) + \frac{k_a}{k_a + k_m} \left[c_a(t = 0)(1 - e^{-\lambda t}) + r_a t - \frac{r_a}{\lambda}(1 - e^{-\lambda t}) \right] \tag{3}$$

In the experiments considered, mixing of the CO_2 pool has gone on for a time $t \gg \frac{1}{\lambda}$ so that the exponential factor becomes $e^{-\lambda t} \ll 1$ and (3) can be rewritten:

$$c \cong \frac{k_m}{k_a + k_m} c_m + \frac{k_a}{k_a + k_m} \left[c_a(t = 0) + r_a t - \frac{r_a}{\lambda} \right] \tag{4}$$

where the measured specific activity of arterial CO_2 at time t is

$$c_a(t) = c_a(t = 0) + r_a t.$$

Equation (4) can be solved algebraically for the unknown specific activity of CO_2 from metabolic sources, in terms of measurable quantities:

$$c_m k_m \cong (k_a + k_m)\, c - k_a c_a + \frac{k_a}{\lambda} r_a \tag{5}$$

The CO_2 flow rates, k_a and k_m, are obtained from measurements of blood flow rates and from chemical analysis of the CO_2 concentration. The specific activities c and c_a, for venous and arterial blood respectively, are obtained by counting the CO_2 of the

VOL. 5 1959/6

blood samples as described. The rate of change of specific activity of arterial CO_2, r_a, is obtained approximately from successive measurements of c_a by dividing the difference in specific activity by the corresponding difference in time. The time constant for the mixing process is obtained from the CO_2 flow constants and an estimate of the effective volume of CO_2 based on brain weight. For convenience, we use flow rates of $^{14}CO_2$ through the various pathways:

From metabolic sources: $R_m = k_m c_m$
To venous blood: $R_v = (k_a + k_m)\, c$
From arterial blood: $R_a = k_a c_a$

Then the correction term because of the change in c_a becomes:

$$\frac{k_a}{\lambda} r_a = k_a \cdot \frac{v}{k_a + k_m} \cdot \frac{d}{dt}(c_a) = k_a \left(\frac{v}{R_v}\right) \frac{d}{dt} \left(\frac{R_a}{k_a}\right) = \frac{v}{R_v} \frac{d}{dt}(R_a)$$

Substituting the above notation, equation (5) becomes:

$$R_m \simeq R_v - R_a + \frac{v}{R_v} \frac{d}{dt}(R_a) \tag{6}$$

Where the flow rate of $^{14}CO_2$ into the brain from metabolic processes is approximately equal to the flow rate out of the brain in venous blood minus the flow rate into the brain in arterial blood, corrected by an amount proportional to the rate of change of $^{14}CO_2$ flow in the arterial blood. This correction term has the effect of taking for the arterial $^{14}CO_2$ flow rate, R_a, a weighted mean value corresponding to a slightly earlier time, because blood leaving the pool at any time contains a mixture of inputs from earlier times.

REFERENCES

ABOOD L. G., GERARD R. W., BANKS J. and TSCHIRGI R. D. (1952) *Amer. J. Physiol.* **168,** 728.

ALLWEISS C. and MAGNES J. (1956) *Abstracts 20th Int. Physiol. Cong.*, p. 21.

ALLWEISS C. and MAGNES J. (1958*a*) *J. Neurochem.* **2,** 326.

ALLWEISS C. and MAGNES J. (1958*b*) *Nature, Lond.* **181,** 626.

BARKER S. B. and SUMMERSON W. H. (1941) *J. biol. Chem.* **138,** 535.

BARKULIS S. S., GEIGER A., KAWAKITA Y. and AGUILAR V. (1960) *J. Neurochem.* **5,** 339.

BELOFF-CHAIN A., CATANZARO R., CHAIN E. B., MASI T. and POCCHIARI F. (1955–56) *Proc. Roy. Soc. B.* **144,** 22.

EISENBERG F. Jr. (1958) In *Liquid Scintillation Counting* (Edited by BELL C. G. Jr. and HAYES F. N.) p. 123, Pergamon Press, New York.

FELLER D. D., STRISOVER S. H. and CHAIKOFF I. L. (1950) *J. biol. Chem.* **187,** 571.

FOLIN O. and MALMROS H. (1929) *J. biol. Chem.* **83,** 115.

GEIGER A. (1958) *Physiol. Rev.* **38,** 1.

GEIGER A. (1959) In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 245. Pergamon Press, London.

GEIGER A., DOMNAS A., KAWAKITA Y. and NEBEL L. (1958) *Fed. Proc.* **17,** 52.

GEIGER A. and MAGNES J. (1947) *Amer. J. Physiol.* **149,** 517.

GEIGER A., MAGNES J. and DOBKIN J. (1954) *Fed. Proc.* **13,** 52.

GEIGER A., MAGNES J. and GEIGER R. S. (1952) *Nature, Lond.* **170,** 754.

GEIGER A., YAMASAKI S. and LYONS R. (1956) *Amer. J. Physiol.* **184,** 239.

GEIGER A., YAMASAKI S. and NEBEL L. (1957) *Fed. Proc.* **16,** 44.

HIMWICH H. E. (1951) *Brain Metabolism and Cerebral Disorders.* Williams, Wilkins, Baltimore.

MITOLO M. (1930) *Physiol. Med. Roma* **7,** 2.

PALLADIN A. V. (1958) In *Radioisotopes in Scientific Research* (Edited by EXTERMANN R. C.) Vol. III. p. 103.
PASSMANN J. M., RADIN N. S. and COOPER J. A. D. (1956) *Analyt. Chem.* **28,** 484.
ROSEN H. (1957) *Arch. Biochem. Biophys.* **67,** 10.
ROSSITER R. F. (1957) In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 355, Pergamon Press, London.
SACHS W. and MOORE S. (1949) *Cold Spr. Harb. Symp. quant. Biol.* **14,** 179.
VLADIMIROV G. E. (1958) In *Radioisotopes in Scientific Research* (Edited by EXTERMANN R. C.) p. 432.
WAELSCH H. (1957) In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 431. Pergamon Press, London.
WINTERSTEIN H. (1918) *Hoppe-Seyl. Z.* **110,** 212.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 339–348. Pergamon Press Ltd. Printed in Northern Ireland

A STUDY ON THE INCORPORATION OF ^{14}C DERIVED FROM GLUCOSE INTO THE FREE AMINO ACIDS OF THE BRAIN CORTEX*

S. S. BARKULIS†, A. GEIGER, Y. KAWAKITA‡ and V. AGUILAR
Research Laboratories, Department of Psychiatry, University of Illinois
College of Medicine

(*Received* 17 *August* 1959)

THE free amino acids constitute a significant fraction of the acid-soluble components of brain (ROBERTS *et al.*, 1950; TALLAN *et al.*, 1954; WAELSCH, 1957). Their composition reflects the importance of glutamic and aspartic acids in brain metabolism since these amino acids and their derivatives (glutamine, asparagine, and acetylaspartic and γ-aminobutyric acids) make up 80 per cent of the total. Any assessment of the major pathways of metabolism in brain must take into account the rate at which this large source of carbon exchanges, replenishes, and is replenished by other intermediary products of metabolism and brain proteins. As part of a continuing study of the pathways of brain metabolism (GEIGER *et al.*, 1960) with uniformly ^{14}C-labelled glucose and the cat brain perfusion method of GEIGER and MAGNES (1947), the amino acids from cortical slices taken at various times during perfusion were isolated and quantitated, and their level of radioactivity was determined. The glutamic and aspartic acids were further separated from the total. The data presented are from perfusion experiments with the brain at rest, under nembutal narcosis, or during convulsions induced by Metrazol.

Most of the experiments reported were also utilized for the study of other aspects of brain metabolism (GEIGER *et al.*, 1960).

EXPERIMENTAL

Details of the experimental conditions during perfusion of cat brains, levels of respiration, rates of perfusion, addition times and amounts of uniformly ^{14}C-labelled glucose, and procedures for brain sampling are described in the preceding paper by GEIGER *et al.* (1960). The findings presented here are from samples taken in a number of the experiments described in that paper and identified by date. The data in the Tables and Figures were calculated per 100 gm brain. The concentration of amino acids was calculated in terms of milligrams of carbon by using a value of 54 mg per millimole for total amino acids determined (based on an average amino acid made up of 4·5 carbon atoms). Specific activities are recorded as counts/min per mg carbon. The level of radioactivity of the various compounds is expressed as the percentage of carbon in each with a specific activity equal to that of the perfusing glucose (columns 6, 9, 12 of Table 1 and Figs. 1 and 2). This is, of course, equivalent to the specific activity of glucose. The carbon dioxide data in the figures are taken from the previous paper.

Chemical

Free amino acids of brain samples were recovered from water homogenates made by suspending 1 gm portions (wet wt) in 9 ml water and grinding in a glass homogenizer. One-tenth volume of 70% perchloric acid was added with mixing. The mixture was allowed to stand 30 min in the cold

* This work was supported by a grant from the National Institute of Neurological Diseases and Blindness, B-413.

† Present address: Research Department, Ciba Pharmaceutical Products, Inc., Summit, New Jersey.
‡ Present address: Department of Neuropsychiatry, Osaka City University Medical School, Osaka, Japan.

room, and the precipitated proteins were sedimented in the centrifuge. The supernatant solution was pipetted off and the protein fraction was washed with 5 ml of 7% perchloric acid in the cold. The supernatant solution recovered from this washing was combined with the first. KOH, 6 N, was added to the combined supernatant solutions, maintained at 0°, until neutrality was reached. The precipitated potassium perchlorate was sedimented in the centrifuge. The neutral supernatant solution was evaporated to dryness under reduced pressure. The dried residue was taken up in 5 ml of 0·1 N-HCl and any residual potassium perchlorate was sedimented and discarded. The sample was now ready for application to a Dowex 50 column.

Dowex 50-X4 resin, 200–400 mesh was washed with HCl and NaOH in the manner prescribed by MOORE and STEIN (1951). In the final step it was washed extensively and suspended in water until used. As needed, columns of 1·1 cm width and 7·5 cm length were poured, and 100–200 ml of 0·1 N-HCl were passed through overnight. On the following day, the sample was applied and allowed to enter the column without pressure. The sample was washed in with two 1·5 ml portions of water, followed by 22 ml of water. The first 30 ml of effluent, containing the neutral and acidic substances, was collected as one fraction. Elution was then begun with 6 N-HCl, and 30 ml were collected as one fraction. This contained the free amino acids.

Glutamic and aspartic acids were separated from the free amino acid fraction by chromatography on columns of Dowex 1-X8 prepared and operated as described by HIRS, MOORE and STEIN (1954). The free amino acid eluates from the Dowex 50 columns in 6 N-HCl were evaporated to dryness under *vacuo*, a small amount of water was added to the dry residue, and the solution was again evaporated to dryness. The latter procedure was repeated once more to ensure a fairly complete removal of residual HCl. The dried amino acid samples were suspended in 2 ml of 0·5 N-acetic acid and applied to the Dowex 1 columns. Fractions were recovered from these columns with the aid of a Model 1205 Automatic Fraction Collector from Research Specialties Company.

The first ninhydrin-positive peak recovered from the Dowex 1 columns contained all the amino acids except glutamine, aspartic and cysteic acids. Glutamic and aspartic acids were eluted as well-separated peaks. Cysteic acid remained on the column, since no special effort was made to recover it. The amino acid content of each peak was quantitated by the ninhydrin procedure as described by ROSEN (1957). The radioactivity of samples was obtained by scintillation counting (Tricarb counter, Model 314, Packard Instrument Company, LaGrange, Illinois). Portions to be counted were pipetted into counting vials and dried under *vacuo*. The samples were dissolved in M-hyamine in methanol prepared according to PASSMAN *et al.* (1956) and scintillator in toluene was added as described by VAUGHN *et al.* (1957).

The first and second brain samples in all experiments were taken within 45 min of the addition of radio-glucose to the perfusion blood. During this period, all animals were kept in a resting state. Metrazol (pentamethylenetetrazol) given to initiate convulsions or nembutal given to initiate narcosis, Table 1, B and C, was started only after the second brain sample was excised.

RESULTS

A general pattern for the appearance of radioactivity in the amino acids is evident (Table 1); it does not vary in any characteristic manner when either narcosis or convulsions supervene. The combined amino acids of the first samples show an appreciable radioactivity, averaging 15–20,000 counts/min per mg C, usually representing 5–10 per cent of the specific activity of the perfused glucose. Although there is frequently an increase of radioactivity in the amino acids of the second sample, this is not pronounced, and in about half of the experiments, there is a small decrease, which is not real, since it is within the limits of analytical and counting errors. However, the amino acids of the third samples generally show a pronounced increase in radioactivity. The specific activity may increase as much as fourfold, as compared with the second sample, and generally not less than two-fold. The interval between the second and third samples varied from 10 to 18 min and this period occurred between 34 and 60 min of perfusion following [^{14}C] glucose addition. The increase in amino acid radio-carbon occurs whether or not Metrazol or nembutal is given

VOL. 5 1959/6

during this period, and its magnitude does not appear to be influenced by these drugs. The amino acids of the last sample show either approximately the same or a somewhat lower level of radioactivity as compared with those of the third. While the decrease when present is not large, it is possibly significant. Thus, in these experiments we find an increasing appearance of radioactivity in the amino acids mostly occurring during the second half hour and then a definite leveling.

The essential features of this pattern for the total amino acids is also found for the glutamic and aspartic acid components. Although the specific activities of these amino acids are higher than the mean for the total amino acid pool, they tend to mirror the changes in the pool. This is not surprising, since the major portion of the free amino acid pool of brain is made up of these amino acids and their derivatives.

A striking finding in these experiments is the remarkable degree of constancy in the amount and composition of the amino acid pool under varying conditions. The range in all experiments for the free amino acid pool (except acetylaspartic acid) is between 25 and 30 μmoles/g brain. This amount of variation was seldom found in any one experiment, however. It was more usual for most of the values in each experiment to be grouped at either extreme of the range. This was true also for glutamic acid, of which the limits of variation were between 8·5 and 10 μmoles/g brain. Only with aspartic acid did a trend indicate a decrease in its concentration as the time of perfusion increased.

The percentage of ^{14}C in the total carbon of CO_2 given off during the 3 resting experiments and 3 of the narcosis experiments increased linearly during the first 30 min, and from then on was maintained at a constant level (Fig. 1). The overall pattern was the same in the Metrazol experiments except for transient changes (see below). The rise in radioactivity of the total amino acid pool in individual experiments (Fig. 1) varied somewhat and in 3 experiments was more rapid between the second and third samples, corresponding to the time when the specific activity of the CO_2 attained its highest level. In one experiment it remained constant throughout the experiment. In none of the experiments, however, was any increase seen in the radioactivity of the total amino acid pool after about 50 to 55 min of perfusion with [^{14}C] glucose. The level obtained was lower than that of the CO_2.

The scatter of glutamic acid values (Fig. 2) from the various samplings does not permit a representative curve to be constructed. Nevertheless, with allowance for an occasional aberrant glutamic acid value, such as that of the third sample (56 min) of the April 30th experiment, a discernible pattern can be seen for each experiment (except May 12) as for total amino acids (Fig. 1). A significant difference, however, is that the average level of radioactivity attained by the glutamic (and aspartic) acids in the last two samplings is higher than that of the total amino acid pool. The average of the eleven values plotted at 50 min and longer is 23·6 per cent and thus, as would be expected, it approximates to the specific activity of the carbon dioxide.

DISCUSSION

The findings presented afford a more detailed view of the extent and rate of the participation of amino acids in the metabolism of the cat brain during perfusion. The manner in which radioactivity originating from uniformly labelled glucose appears in the amino acids, while consistent with known metabolic interrelationships in the main, also has a number of features which could not have been anticipated.

Table 1.—Amino acids in the cortex of perfused cat brain

per 100 gram brain

Experiment	Perfusion with [^{14}C] glucose (min)	Glucose specific activity (counts/min per mg C)	TOTAL AMINO ACIDS			GLUTAMIC ACID			ASPARTIC ACID		
			Present as carbon (mg)	Specific activity (counts/min per mg C)	Carbon derived from glucose* (%)	Present as carbon (mg)	Specific activity (counts/min per mg C)	Carbon derived from glucose* (%)	Present as carbon (mg)	Specific activity (counts/min per mg C)	Carbon derived from glucose* (%)
					A. Resting						
April 30	28	354,000	129·5	15,700	4·44	49·5	24,100	6·8	7·92	20,700	5·9
	44	354,000	140·0	23,900	6·95	50·0	42,300	12·0	8·35	47,300	13·4
	56	354,000	136·5	70,000	19·80	51·0	135,000	38·2	7·88	126,500	35·7
	70	354,000	134·0	74,100	21·00	54·6	107,000	30·2	7·92	95,500	26·8
May 12	26	262,000	162	20,300	7·72	54·7	34,000	13·0	11·5	30,400	11·5
	40	262,000	169	25,200	9·61	57·4	38,200	14·5	10·2	32,300	12·4
	54	262,000	168	25,100	9·60	60·0	36,800	14·1	9·6	33,000	12·6
	68	262,000									
June 10	22	328,000	155	12,400	3·78	60	17,500	5·32	14·5	15,000	4·62
	40	328,000	149	24,400	7·45	60	32,700	9·78	12·2	29,500	7·6
	56	328,000	146	58,200	17·72	60	62,200	19·0	10·7	79,400	17·9
	56	328,000	151	43,000	13·0	64	56,800	17·2	9·6	57,000	15·1
					B. Narcosis						
June 4	28	246,000	162	18,800	7·62	64·5	30,300	12·3	10·3	36,900	15·0
	42	246,000	165	23,700	9·58	56·2	42,200	17·2	12·5	33,000	13·4
	46	nembutal added to perfusion blood									
	58	246,000	159	52,000	21·1	63·6	79,600	32·4	9·88	94,500	40·0
	70	246,000	159	45,500	18·4	55·4	74,800	30·3	7·9	93,500	42·0

VOL. 5
1959/6

OL.
5
9/60

May 19	18	283,000	143	19,650	6·94	60.	23,300	8·25	12·1	23,500	8·26
	38	283,000	139	18,200	6·42	48·2	24,000	8·46	9·35	19,300	6·85
	40	nembutal added to perfusion blood									
	54	283,000	140	31,700	11·11	49·0	45,300	16·0	9·80	47,100	16·6
	68	283,000	143	25,500	8·95	48·0	39,700	14·1	7·30	41,700	14·8
May 5	26	300,000	135	42,000	14·0	62·8	42,300	14·1	11·1	41,700	13·9
	42	300,000	134	39,800	13·3	59·5	39,600	13·2	8·94	47,200	15·8
	50	nembutal added to perfusion blood									
	60	300,000	131	78,500	26·2	60·5	84,600	28·3	8·64	109,000	36·4
	76	300,000	130	73,000	24·3	57·0	86,000	28·8			
April 23	20	252,000	156	15,750	6·26	60·3	23,000	9·15	9·9	20,300	8·1
	34	252,000	156	15,100	5·97	63·5	18,200	7·23	11·2	18,600	7·3
	42	nembutal added to perfusion blood									
	50	252,000	159	39,200	15·6	66·5	56,700	22·5	7·6	56,700	22·5
	62	252,000	162	41,700	16·5	66·0	65,200	26·0	6·5	75,000	29·9
					C. Convulsions						
April 7	18	410,000	152	5,520	1·35	54·5	5,700	1·4			
	34	410,000	150	13,900	3·40	61·2	94,300	3·5	11·7	13,700	3·33
	38	410,000		METRAZOL CONVULSIONS							
	44	410,000	166	63,900	15·50	63·0	77,500	18·9			
	52	410,000	159	53,800	13·13	61·9	68,000	16·6	11·7	64,800	15·8
March 3	20	345,000	144	31,600	9·0	60·0	47,100	13·7	10·2	42,100	12·3
	36	345,000	159	70,000	20·3	54·2	98,500	28·5	11·4	73,800	21·3
	40			METRAZOL CONVULSIONS							
	46	345,000	134	86,500	25·00	54·0	107,000	30·9	10·6	114,000	33·0
	52	345,000	147	99,300	28·8	56·3	148,000	43·0	9·75	156,000	45·4
	58	345,000	132	111,000	32·8	52·6	147,000	42·6	10·60	145,000	42·1

* Equivalent to (SA of amino acid/SA of glucose) × 100

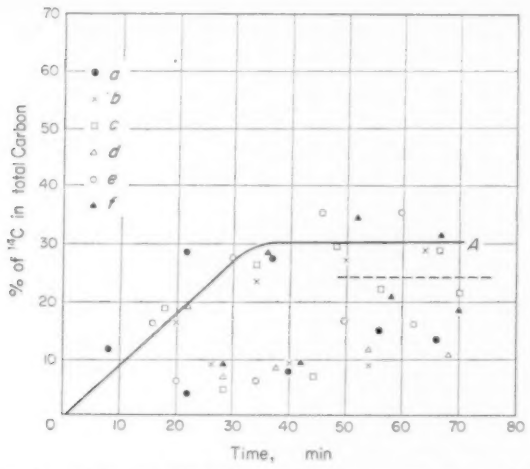


FIG. 1.—The plots of the individual values of ^{14}C incorporation into CO_2 from the various experiments fall into a pattern which can be depicted by the curve A. Since the absolute values of ^{14}C incorporation into the amino acid varied from experiment to experiment, it is not possible to express the curve of ^{14}C incorporation by averaging all the values from different experiments and, therefore, only the individual values are plotted. The broken line separates the plotted values referable to curve A from those which represent ^{14}C in amino acids. If the points representing ^{14}C incorporation into amino acids are viewed for each experiment separately, it becomes evident that radioactivity increases only during the first 53 min of perfusion and then levels off in each experiment except (c)
(a) Exp. June 10, (b) May 12; (c) April 30; (d) May 19; (e) April 23; (f) June 4.

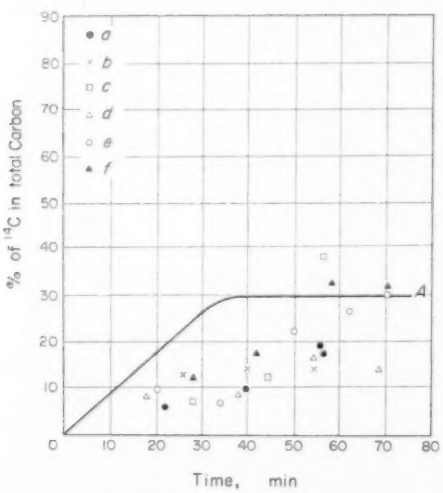


FIG. 2.—Time course of incorporation of ^{14}C derived from glucose into glutamic acid and respiratory carbon dioxide. Curve A as in Fig. 1. Values plotted for glutamic acid are from Table 1. The symbols are the same as in Fig. 1.

By transaminase action it would be reasonable to expect a rapid exchange and equilibration of alanine, aspartic acid, and glutamic acid carbon with that of pyruvate and of the tricarboxylic acids. From these amino acids radiocarbon would gain entry into a number of others by further familiar reactions, e.g. transamination, amination,

VOL.
5
1959/6

decarboxylation, etc. We know that the amino acid pool* (approximately 150 mg C/100 g brain) is very much larger than that made up of pyruvate and of the intermediates of the tricarboxylic acid cycle (probably less than 1 mg/100 g brain). Since glycolysis represents the major, if not exclusive, pathway for breakdown of glucose in brain, one third of the respired CO_2 is contributed from the carbons 3 and 4 of glucose by way of pyruvate, and two thirds come by way of the tricarboxylic acid cycle (glucose carbons 1, 2, 5 and 6). If we assume that the only source of carbon exchanging with pyruvate and the tricarboxylic acid cycle intermediates is the amino acid pool and that such exchange occurs at equal and non-limiting rates in relation to the turnover of the former, then the specific radioactivity of the respired CO_2 should closely parallel and be almost equal to that of all these metabolites. This is evidently not the case, since the acquisition of radioactivity by the amino acid pool and more particularly by glutamic acid lags behind and does not come up to the level in carbon dioxide during the course of 60–70 min of perfusion (Figs. 1 and 2). These findings are not surprising, however, since the principal amino acid with which pyruvate would be expected to exchange rapidly, namely alanine, makes up a small fraction of the amino acid pool (less than 5 per cent) whereas glutamic and aspartic acids and their derivatives, which would exchange with cycle intermediates, comprise the major part of the free amino acids. Consequently, the dilution of pyruvate carbons by amino acid carbon may, for our discussion, be considered negligible in comparison with the dilution of the cycle intermediates from this source. That portion of the respired carbon dioxide which comes from the oxidative decarboxylation of pyruvate should, therefore, contain a higher radioactivity than that arising from the tricarboxylic acid cycle until such time as the cold carbon from the amino acids exchanging with cycle intermediates has been replaced. Consequently, the slower rise of amino acid radioactivity in relation to that of respired CO_2 can be readily understood. The specific activity of glutamic and aspartic acids should be a fairly good gauge of the activity of the tricarboxylic acid cycle components.

An important feature of the data presented in Figs. 1 and 2 is not, however, explained by the foregoing considerations, namely, that the radioactivity of both the respired CO_2 and the amino acids increases during roughly the first half of the perfusion (although that of the amino acids not as linearly as of the CO_2) and then remains at a constant level during the balance of the perfusion time. If one again assumes a simple situation in which carbon from exogenous glucose exclusively (or almost so) replenishes the glycolytic and cycle intermediates and in which the amino acids represent the only carbon pool which rapidly equilibrates with these carbons, then at the rates in which glucose is being taken up by the brain, and with acceptance of certain values for the concentrations of the glycolytic intermediates, we can predict the pattern and some of the dimensions of the rates of increase of radioactivity in carbon dioxide and the amino acids. Under these circumstances, the level of radioactivity in carbon dioxide would increase at a fairly rapid and constant rate during the period in which the pre-existing pool of glucose and glycolytic intermediates was being replaced by [^{14}C] glucose, then at a slower but nonetheless appreciable rate during the period in which the amino acid carbon was equilibrating with tricarboxylic acid cycle intermediates. At the point of near-completion of the exchange of amino acid carbon, the radioactivity of the respired CO_2 would be expected to

* Reference to the amino acid pool in this paper does not include acetylaspartic acid.

rise at a very slow rate, reflecting the rate of entry of cold carbon from the combined turnover of brain lipid, protein, and nucleic acids.

In most of these experiments, glucose carbon is taken up by the brain at a rate of 4–5 mg C/min per 100 g brain. A fairly constant value of 2·6 mg C/min (which equals the rate at which CO_2 carbon is given off) is available for supporting brain metabolism whereas the balance is put back out into the perfusing blood as lactic acid. At this rate of [^{14}C] glucose input, it has been estimated (GEIGER *et al.*, 1960) that the pre-existing brain glucose would be completely replaced in less than 5 minutes. Since the glycolytic intermediates make up a total pool of about 32 mg C (ABOOD *et al.*, 1952), it can be calculated that within 25 min after the addition of [^{14}C] glucose to the perfusion blood these intermediates would have 90 per cent or more of the specific activity of the glucose. During this period, the radioactivity of the respired carbon dioxide would increase until by 25 min it should be 30 per cent of the glucose (at a minimum) since one third of the CO_2 carbon would be coming from pyruvate which at this point would have a specific activity comparable to glucose. Furthermore, during the first 60 min of perfusion with [^{14}C] glucose, approximately 100 mg of ^{14}C would have entered the cycle (carbons 1, 2, 4, 5) and exchanged with 150 mg amino acid carbon, thus raising the level of radioactivity of amino acid carbon to about 50 per cent of that of glucose carbon. There would, therefore, be a steady rise in the radioactivity of the respired carbon dioxide from a minimal value of about 30 per cent at 25 min to 65 per cent at 60 min.

It is quite apparent (Figs. 1 and 2) that neither the carbon dioxide nor the amino acids conform to this pattern. The data appear to us to be best understood by assuming that a source of cold carbon is feeding into and forming glycolytic and tricarboxylic acid intermediates at a rate which is almost as large as the one in which they are formed from exogenous glucose taken up by the brain. The source of this cold carbon is at present unknown. The total uptake of ^{14}C into lipids, proteins and nucleic acids (GEIGER *et al.*, 1958) during this period is only a fraction of the amount of cold carbon which is forming carbon dioxide. The major portion of the radioactivity which is taken up as glucose by the brain and does not appear in the respired carbon dioxide is present in the acid-soluble fraction which probably includes the glycolytic and possibly the tricarboxylic acid intermediates and increases considerably during perfusion. Although much less likely, it may be that there exists a large pool of acid soluble components in normal brain which rapidly exchanges with glycolytic and cycle intermediates and that the pool, by virtue of its size, acquires an insignificant specific activity during the course of 60–70 min of perfusion.

Narcosis, while decreasing the rate of respiration, does not appreciably change the relative rates at which carbon from perfused [^{14}C] glucose and endogenous cold carbon contribute to the respiratory carbon dioxide and to the pattern of labelling of the amino acids as seen in the resting state. Perhaps it is more surprising that convulsions do not markedly change the labelling pattern in the amino acids, even though during the convulsive period there is an enhanced respiratory rate and a marked though temporary decrease in the radioactivity of the respired carbon dioxide. It might have been expected that this decreased radioactivity of the carbon dioxide, reflecting a greater contribution of cold endogenous carbon, should be accompanied by a decrease in the radioactivity of the amino acids. Since this did not happen, we can only speculate that during the brief period of heightened respiration, resulting in a

VOL.
5
1959/6

transient decrease in the specific activity of respired CO_2, the concentration of the tricarboxylic acid intermediates may have also increased several fold, permitting a much more rapid exchange rate with amino acids. Consequently, although the specific activity of the cycle intermediates decreased during this time, the more rapid exchange with amino acids than in the resting state may have been sufficient actually to introduce more ^{14}C into the latter. The data presented for the Metrazol experiments illustrate the difficulty, even at the present state of our knowledge of intermediary metabolic pathways and relationships, in rationalizing, much less predicting, how various metabolites will be altered when the overall metabolism of a major organ is changed. Obviously, we need to know considerably more about the relative rates at which different pools of intermediates are exchanging during different levels of organ activity. The problems encountered are well illustrated by the earlier work of RAFELSON and WINZLER *et al.* (1951; 1952) who compared minces of day-old mouse brain with day-old and adult mouse brain *in vivo* for ability to incorporate ^{14}C from glucose into amino acids. A number of studies have appeared in the last few years from the WAELSCH group (LAJTHA *et al.*, 1957, 1959; LAJTHA, 1959) on the related problem of the rates of exchange between amino acids of the blood and of the brain.

The concentrations of amino acids in cat brain cortex and of the glutamic and aspartic acid fractions are consistent with those reported by TALLAN *et al.* (1954) for the whole brain. In several experiments, the concentration, distribution of glutamic and aspartic acids, and specific activity of the amino acids of whole brain homogenates approximated to the corresponding values from the cortex. Only preliminary data are available concerning the concentrations and radioactivity of acetylaspartic acid (TALLAN *et al.*, 1956) at various times in these experiments. These suggest that the aspartic acid moiety of this conjugated compound does not exchange to any appreciable extent with free aspartic acid. Work is in progress to obtain further information concerning this and some of the other amino acids during perfusion.

Preliminary data on the separation of the amino acid fraction remaining after the removal of glutamic and aspartic acids indicate that glutamine, serine, alanine, and glycine attain radioactivities comparable to those of glutamic and aspartic acids, whereas the essential amino acids leucine, phenylalanine, and lysine do not become radioactive.

SUMMARY

The free amino acids of cortical samples taken from cat brains during the course of perfusion with a simplified blood containing uniformly ^{14}C-labelled glucose were isolated and quantitated, and their radioactivity was determined. The glutamic and aspartic acid components were further separated and analysed. Our goal was to obtain information which would aid in assessing the role of amino acids in relation to the metabolism of glucose under resting conditions, during Metrazol convulsions, and following narcosis induced with nembutal. The radioactivity of the respired carbon dioxide and the rates of glucose uptake in these experiments, reported in detail in a preceding paper, in conjunction with the data presented, permit a more detailed view of the relative contribution of exogenous glucose and carbon from endogenous sources to brain respiration.

The radioactivity in the amino acids rose irregularly during the first 50 min of perfusion, in some experiments with a sharp jump between 30 and 50 min; it reached a

specific activity of 15–18 per cent that of glucose and then remained constant in all experiments. Glutamic and aspartic acids showed the same picture but levelled at 23–24 per cent of the specific activity of glucose. Respiratory carbon dioxide increased during the first 30 min of perfusion and then remained constant at 29–32 per cent of the specific activity of glucose.

This pattern of entry of ^{14}C into amino acids was similar for resting brain, during narcosis, and during convulsions. Implications of these data are discussed. A major conclusion is that endogenous carbon sources were contributing to form glycolytic and tricarboxylic acid intermediates at a rate which was almost as great as that by which they were being formed from exogenous glucose.

REFERENCES

ABOOD L. G., GERARD R. W., BANKS J. and TSCHIRGI R. D. (1952) *Amer. J. Physiol.* **168,** 728.
GEIGER A., DOMNAS A., KAWAKITA Y. and NEBEL L. (1958) *Fed. Proc.* **17,** 52.
GEIGER A., KAWAKITA Y. and BARKULIS S. S. (1960) *J. Neurochem.* **5,** 323.
GEIGER A. and MAGNES J. (1947) *Amer. J. Physiol.* **149,** 517.
HIRS C. H. W., MOORE S. and STEIN W. H. (1954) *J. Amer. Chem. Soc.* **76,** 6063.
LAJTHA A. (1959) *J. Neurochem.* **3,** 358.
LAJTHA A., BERL S. and WAELSCH H. (1959) *J. Neurochem.* **3,** 322.
LAJTHA A., FURST S., GERSTEIN A. and WAELSCH H. (1957) *J. Neurochem.* **1,** 289.
MOORE S. and STEIN W. H. (1951) *J. biol. Chem.* **192,** 663.
PASSMAN J. M., RADIN N. S. and COOPER J. A. D. (1956) *Analyt. Chem.* **28,** 484.
RAFELSON M. E., WINZLER R. J. and PEARSON H. E. (1951) *J. biol. Chem.* **193,** 205.
ROBERTS E., FRANKEL S. and HARMAN P. J. (1950) *Proc. Soc. exp. Biol., N.Y.* **74,** 383.
ROSEN H. (1957) *Arch. Biochem. Biophys.* **67,** 10.
TALLAN H. H., MOORE S. and STEIN W. H. (1954) *J. biol. Chem.* **211,** 927.
TALLAN H. H., MOORE S. and STEIN W. H. (1956) *J. biol. Chem.* **219,** 257.
VAUGHN M., STEINBERG D. and LOGAN J. (1957) *Science* **126,** 448.
WAELSCH H. (1957) In *Metabolism of the Nervous System* (Edited by RICHTER D.) p. 431. Pergamon Press, London.
WINZLER R. J., MOLDAVE K., RAFELSON M. E. and PEARSON H. E. (1952) *J. biol. Chem.* **199,** 485.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 349–353. Pergamon Press Ltd. Printed in Northern Ireland

THE APPLICATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE DIAPHORASE METHODS IN A STUDY OF DORSAL GANGLIA AND SPINAL CORD*

T. SAMORAJSKI
Department of Anatomy, Ohio State University, Columbus, Ohio

(*Received* 4 *September* 1959)

FOLLOWING the introduction of blue tetrazolium salt (BT) by SELIGMAN and RUTENBURG (1951) for the demonstration of succinic dehydrogenase, FARBER *et al.* (1956) succeeded in using this reagent in developing a histochemical method for localization of the diaphorases. With the preparation of *p*-nitro-phenyl substituted ditetrazole (Nitro BT) by TSOU *et al.* (1956), several of the shortcomings prevalent in the original method of FARBER and his associates were overcome and satisfactory cytochemical localization was obtained.

Recently, SCARPELLI, HESS and PEARSE (1958) were able to obtain localization of diphosphopyridine nucleotide (DPN) diaphorase and triphosphopyridine nucleotide (TPN) diaphorase in mitochondria by a method involving metal chelation of 3:5-diphenyl-2-(4:5-dimethylthiazol-2-yl)tetrazolium bromide (MTT) and utilizing reduced coenzymes DPNH and TPNH as substrates. Methods utilizing both Nitro BT and MTT have now been developed to the extent where a considerable number of pyridine nucleotide linked dehydrogenases can be demonstrated. Diaphorase activity, however, though easily demonstrable in many different types of tissues, appears not to have been adequately studied in the nervous system.

The present paper is concerned with the localization of diaphorase activity in rat dorsal ganglia and spinal cord. Both Nitro BT and MTT were used in the procedure and fresh as well as fixed frozen-cut sections were employed. The results indicate that there is a characteristic distribution of diaphorase activity in these tissues and an accurate localization of certain of the neural elements can be obtained.

MATERIALS AND METHODS

The present studies were carried out with fresh and fixed frozen-cut sections of rat dorsal ganglia and spinal cord obtained from the area of the lumbosacral enlargement ($L_4 - S_3$). Intact dorsal ganglia were removed from the animals and placed on specimen holders for immediate freezing and sectioning with a Sartorius freezing microtome. Spinal segments about 2 to 4 mm thick were rapidly frozen with dry ice and placed in a cold (−20°) cryostat for sectioning. Cut sections (14 μ) were directly transferred to an incubating medium containing either Nitro BT or MTT.

To determine the effect of fixation on DPN-diaphorase activity, additional dorsal ganglia and spinal cord segments were immersed for 2 hr in one of the following cold (4°) fixatives previous to sectioning with a freezing microtome.

1. Absolute acetone.
2. 2% Calcium–formalin made by neutralizing 10% formalin with sodium acetate to a pH of 7 and adding $CaCl_2$ to make up a 2% solution.

* This investigation was supported by a research grant (B-1299) from the National Institute of Neurological Disease and Blindness of the National Institutes of Health, Public Health Service.

3. Chloral-hydrate–formalin (BAKER *et al.*, 1958). Chloral-hydrate–glycerine jelly embedding was occasionally utilized with spinal cord segments.

The incubating media used in these experiments were prepared according to the directions of SCARPELLI *et al.* (1958) with minor modifications. The molarity of DPNH in the substrate was reduced to 0·1 M and tris buffer was used at a pH of 7·2. Under these conditions, the possibility of false localization is less likely to occur (ZIMMERMANN and PEARSE, 1959).

Crystalline ribonuclease (1 mg/ml made up in either glass distilled water, or in 0·5 M-tris buffer at a pH of 7·2) was used to study the effect of ribonuclease extraction on diaphorase localization. Both fresh and fixed frozen-cut sections were employed. Tissue sections prepared for histologic control of the ribonuclease reaction were stained in dilute thionine (1 : 3000 parts of water) for 20 min at 58°.

The localization of sulphydryl (SH) and disulphide (S-S) groups was studied by the tetrazolium method of BARRNETT and SELIGMAN (1954). Both neotetrazolium chloride and Nitro BT were used on fresh and fixed frozen-cut sections. Incubation was carried out at room temperature for a period of 2–8 hr.

RESULTS

The opinion that the diaphorase system is capable of visualizing differences among various cells and even distinguishing different functional states of the same cell type has previously been expressed by HESS *et al.* (1958). Our results, obtained with fresh dorsal ganglia and spinal cord stained by both Nitro BT and MTT methods, are in agreement with this statement. All neurons of the dorsal ganglia show cytoplasmic localization of DPN-diaphorase activity. Various degrees of activity are, however, commonly observed among the different neurons of any single ganglion with the largest cells frequently exhibiting the weakest activity (Figs. 1–4).

In the spinal cord, the cytoplasmic reaction is even more selective, staining primarily anterior horn cells located in the ventromedial and ventrolateral groups of the ventral horn (Fig. 10).

Enzymic localization with Nitro BT and MTT in fresh tissue sections

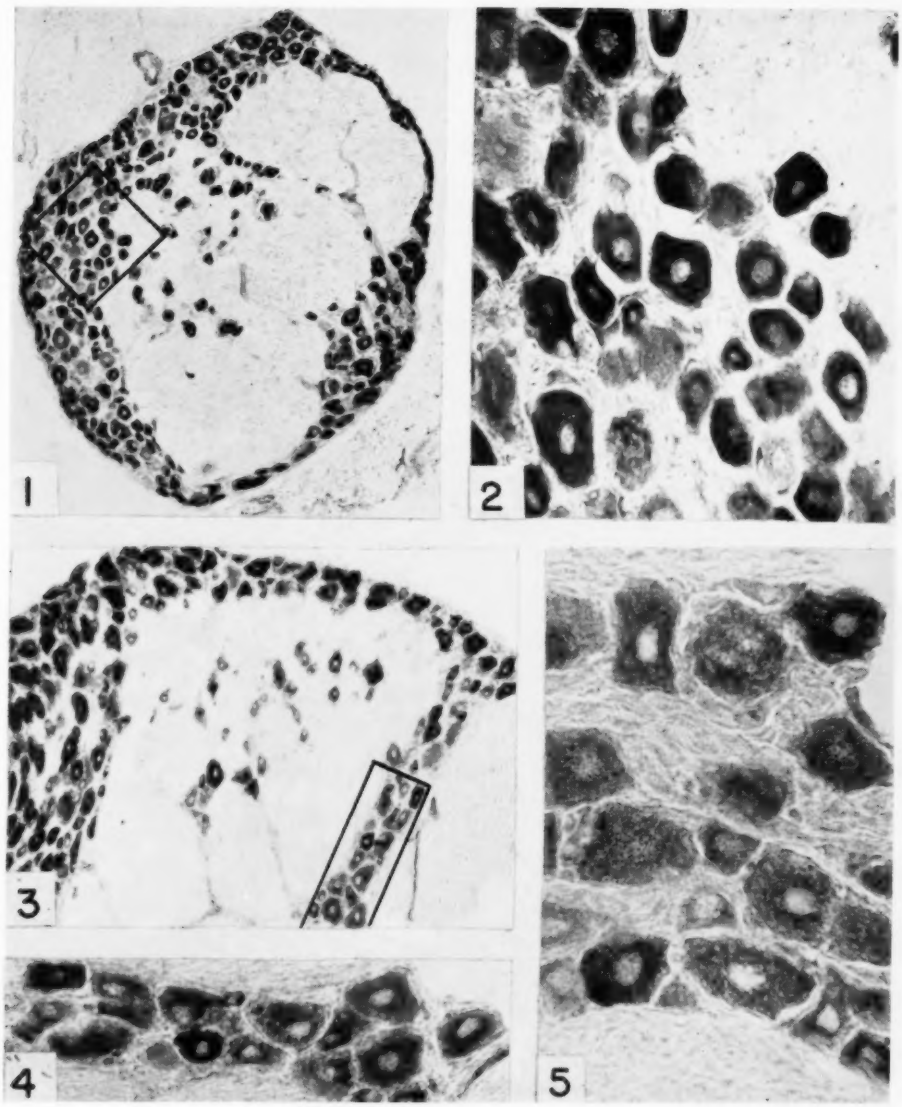
Significant differences in cytoplasmic diaphorase localization are apparent when results obtained with nitro BT are compared with those obtained by the MTT-cobalt method. When Nitro BT is used in the incubating medium, a diffuse though intense cytoplasmic localization is observed in the dorsal ganglia (Fig. 2) as well as in certain of the ventral horn cells (Fig. 11). These results suggest that the use of Nitro BT facilitates both 'microsomal' and mitochondrial localization. With MTT, however, sites of activity appear in the form of small deposits of formazan, ranging from 0·2 to 0·4 μ in diameter (Figs. 5 and 14). These results with MTT are suggestive of mitochondrial diaphorase localization. Neuropil in the spinal cord is slightly reactive and can be differentiated from the white matter with either Nitro BT or MTT.

To rule out the possibility of obtaining a non-enzymic tetrazole reduction in our preparation, fresh sections were incubated in solutions containing all of the reagents of the incubation mixture except the substrate DPNH. With this procedure, reduction of Nitro BT and MTT was never demonstrated unless incubation was extended to 18 or more hr, in which instance only a slight deposit of formazan pigment was observed.

Effect of fixation on DPN-diaphorase localization

Several advantages can be gained by employing a fixative which precipitates protein, producing adequate tissue fixation and still causing no inactivation of

VOL. 5 1959/6



All figures shown are for rat dorsal ganglia or spinal cord obtained from the area of the lumbo-sacral enlargement.

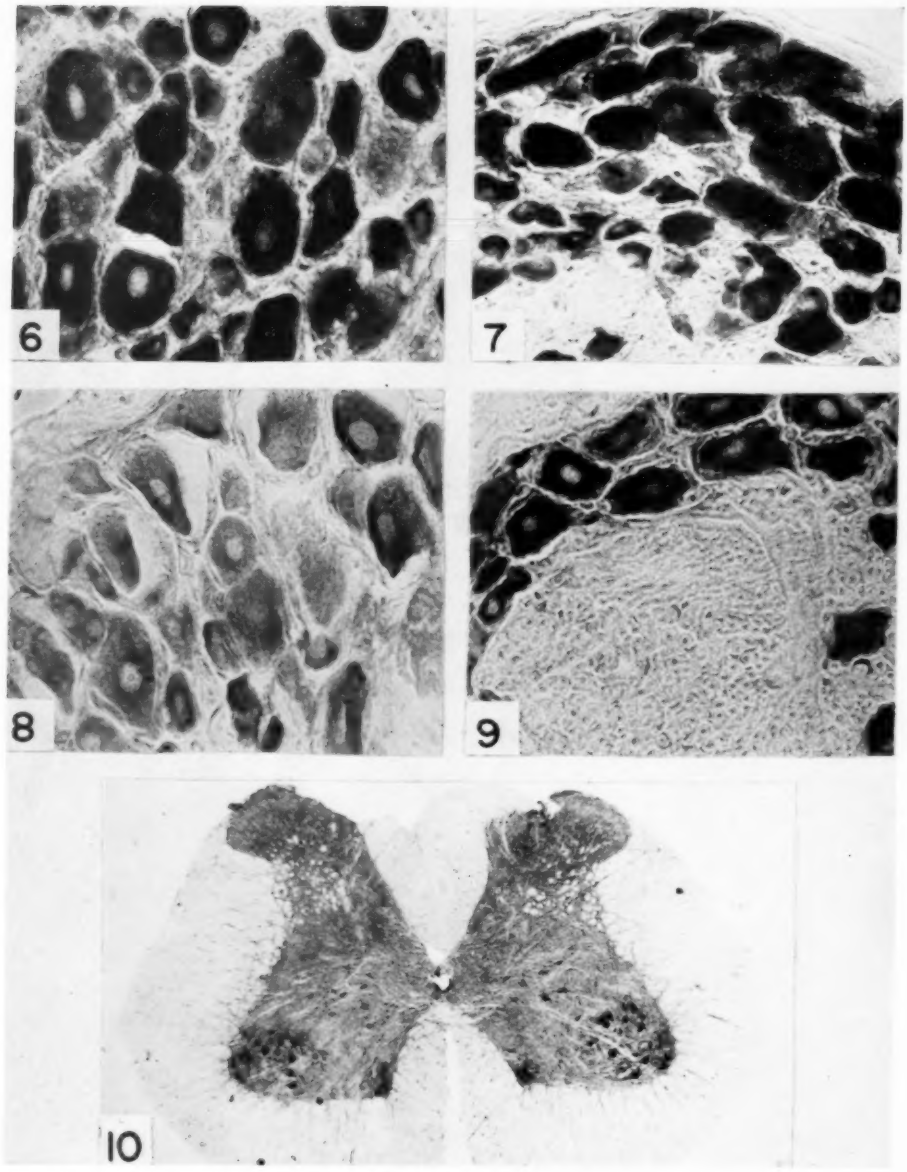
FIG. 1.—Fresh dorsal ganglion, Nitro BT. Note differences in the intensity of the cytoplasmic DPN-diaphorase reaction among the various neurons. ×70.

FIG. 2.—Enlargement of structures within the rectangle of the previous figure. Diffuse localization of formazan pigment can be seen in the cytoplasm. ×280.

FIG. 3.—Fresh dorsal ganglion, MTT. Variable degrees of neuronal diaphorase activity are also demonstrated with this method. ×70.

FIG. 4.—Same as Fig. 3, enlarged to show dot-like deposits of formazan in the cytoplasm of neurons within the rectangle of the previous figure. ×230.

FIG. 5.—Fixed (2% calcium–formalin) dorsal ganglion stained for diaphorase activity with MTT. Dot-like deposits of formazan in the cytoplasm are suggestive of mitochondrial localization. ×320.



VOL.
5
1959/6

FIG. 6.—Fixed (2% calcium–formalin) dorsal ganglion, Nitro BT. The cell bodies show an intense though diffuse localization of diaphorase activity. ×280.

FIG. 7.—Fixed (chloral-hydrate–formalin) dorsal ganglion, Nitro BT. Note preservation of cytoplasmic diaphorase activity. ×280.

FIG. 8.—Fixed (acetone) dorsal ganglion, Nitro BT. Only a slight degree of activity is demonstrated. ×280.

FIG. 9.—Section of fixed (2% calcium–formalin) dorsal ganglion incubated in RNA-ase previous to staining for diaphorase activity with Nitro BT. Note that RNA-ase has no appreciable effect on the cytoplasmic localization of diaphorase. ×280.

FIG. 10.—Fixed (2% calcium–formalin) spinal cord, Nitro BT. Localization of diaphorase activity in anterior horn cells and gray matter neuropil. ×17.

enzyme. In addition to making the enzyme protein more insoluble and consequently reducing the possibility of diffusion, laborious cryostat procedures can frequently be avoided. It has recently been shown that fixation of tissues in a chloral-hydrate–formalin mixture succeeds in preserving β-glucuronidase activity for a considerable period of time (FISHMAN and BAKER, 1956). Alkaline phosphates, esterase, and lipase are similarly preserved in the presence of chloral-hydrate–formalin (BAKER *et al.*, 1958). Accordingly, the effects of a variety of fixatives on DPN-diaphorase localization in nerve tissue were studied and the following results were noted.

1. *Chloral-hydrate–formalin fixation.* Fixation of dorsal ganglia and spinal cord tissue in chloral-hydrate–formalin for 2 hr prior to sectioning and incubation produces a more intense reaction than is obtained with similarly processed unfixed tissues. The only other appreciable difference shown is the somewhat granular appearance of formazan in the cytoplasm of dorsal ganglia cells (Fig. 7) and in the cytoplasm and dendritic processes of stainable anterior horn cells (Fig. 13).

On several occasions, we have embedded chloral-hydrate–formalin fixed spinal cord segments in chloral-hydrate–glycerine jelly to facilitate sectioning. No differences were noted in the results by the addition of this step.

2. *Effect of 2% Calcium–formalin fixation on diaphorase localization with nitro BT (Figs. 6 and 12).* Figs. 5 and 14 were obtained from formalin fixed tissue by the MIT–cobalt method. When these results are compared with fresh (unfixed) stained sections, it is apparent that fixation with 2% calcium–formalin for 2 hr previous to staining also produces an intensification of the reaction. Even after 24 hr of fixation, positive results can still be obtained in both dorsal ganglia and spinal cord although the intensity of the reaction is considerably decreased.

3. *Absolute acetone.* Since cold acetone is a commonly employed 'fixative' in enzyme histochemistry, it was of interest to test the influence of this solution on DPN-diaphorase activity. The results clearly indicate that activity is almost completely lost in dorsal ganglia (Fig. 8) and also in the spinal cord after only 2 hr of exposure to cold acetone.

The influence of ribonuclease on the DPN-diaphorase reaction

Our results with Nitro BT suggest the presence of an extramitochondrial pathway which is supposedly localized in microsomes. Since it is known that microsomes contain a high proportion of ribonucleic acid (RNA), incubation of sections in a solution of crystalline ribonuclease might alter extramitochondrial localization of diaphorase. Although treatment of ganglia and spinal cord sections with ribonuclease results in a loss of stainability of Nissl's substance with thionine, the DPN-diaphorase reaction is not significantly affected (Fig. 9). These results indicate that microsomal RNA is not the contributing factor, but it does not rule out the possibility that 'endoplasmic reticulum' or some other structural component is responsible for the diffuse reaction.

Miscellaneous

ZIMMERMANN and PEARSE (1959) have been able to show that tissue sulphydryl groups are able to reduce exogenous DPN (or TPN) at alkaline pH levels and produce a 'nothing dehydrogenase' effect. To determine whether or not this phenomenon was contributing to our results, sections of dorsal ganglia and spinal cord were

examined for both sulphydryl (SH) and disulphide (S–S) groups. However, thiol activity was never indicated with neotetrazolium chloride, and only a few neurons in the dorsal ganglia showed a very slight activity after 8 hr of incubation in a medium containing Nitro BT. Thus, our results are not dependent on the presence of sulphydryl groups.

COMMENTS

There seems to be little doubt that alterations of the mitochondrial organization can be elicited by a variety of factors including freezing and thawing, anisotonicity, and pH (HESS *et al.*, 1958), narcotics (ERNSTER *et al.*, 1955), and some ions (ERNSTER and LÖW, 1955), etc., which result in a release of soluble cofactors affecting the respiratory capacity of the system. It has recently been shown that there are two alternative oxidative pathways for DPNH which are probably catalysed by two different forms of mitochondrial DPNH–cytochrome *c* reductase activity. One pathway is phosphorylative and involves mitochondrial dehydrogenase. The other is non-phosphorylative, is extramitochondrial and supposedly localized in microsomes. It is further believed that diaphorase alone is responsible for the reduction of tetrazolium salts, and the localization of formazan in tissue sections specifically represents intracellular sites of activity. Thus, intramitochondrial activity is represented as dot-like deposits of formazan, whereas diffuse formazan staining observed in cytoplasm supposedly represents microsomal respiratory activity.

The results of the experiments reported here demonstrate that the distribution pattern of diaphorase activity in tissue sections with the MTT-cobalt method is different from, and usually more restricted than the distribution of diaphorase revealed by Nitro BT. Since Nitro BT has a lower reduction (redox) potential than MTT (PEARSE), it is quite possible that Nitro BT is capable of demonstrating sites of lower activity than is MTT, and thus produces a more intense and diffuse localization. These findings are at variance with the results of HESS *et al.* (1958), who were unable to demonstrate an extramitochondrial oxidative pathway with Nitro BT, but are in accord with the views of NACHLAS *et al.* (1958) concerning diffuse patterns of cytoplasmic TPN-diaphorase activity.

It has previously been indicated that some metallic ions are capable of effecting mitochondrial organization. It is thus possible that the cobalt used for the metal chelation of formazan with the MTT-cobalt method is not without some effect on the enzymic or osmotic integrity of the mitochondria which favours an intramitochondrial localization of activity. Although it has been shown that Ca^{++} causes a marked decrease in respiration (ERNSTER, 1956), fixation of tissues in formalin containing Ca^{++} results in a marked increase of activity when Nitro BT is used, and causes no significant alteration in activity with MTT. The bearing of these phenomena on the cytoplasmic localization of diaphorase activity requires further investigation.

The present study clearly indicates that there are differences in the level of DPN-diaphorase activity existing within a comparatively limited group of neurons in the dorsal ganglia and spinal cord of the rat. Similar alterations in cholinesterase activity determined by quantitative microchemical methods have been described for sympathetic and spinal ganglia (GIACOBINI, 1957*a*, *b*) and anterior horn cells of the spinal cord (GIACOBINI and HOLMSTEDT, 1958). Preliminary experiments in our laboratory indicate that neurons undergoing chromatolysis induced by transection of the sciatic nerve, show a considerable decrease in DPN-diaphorase localization. Thus, the

VOL.
5
1959/6

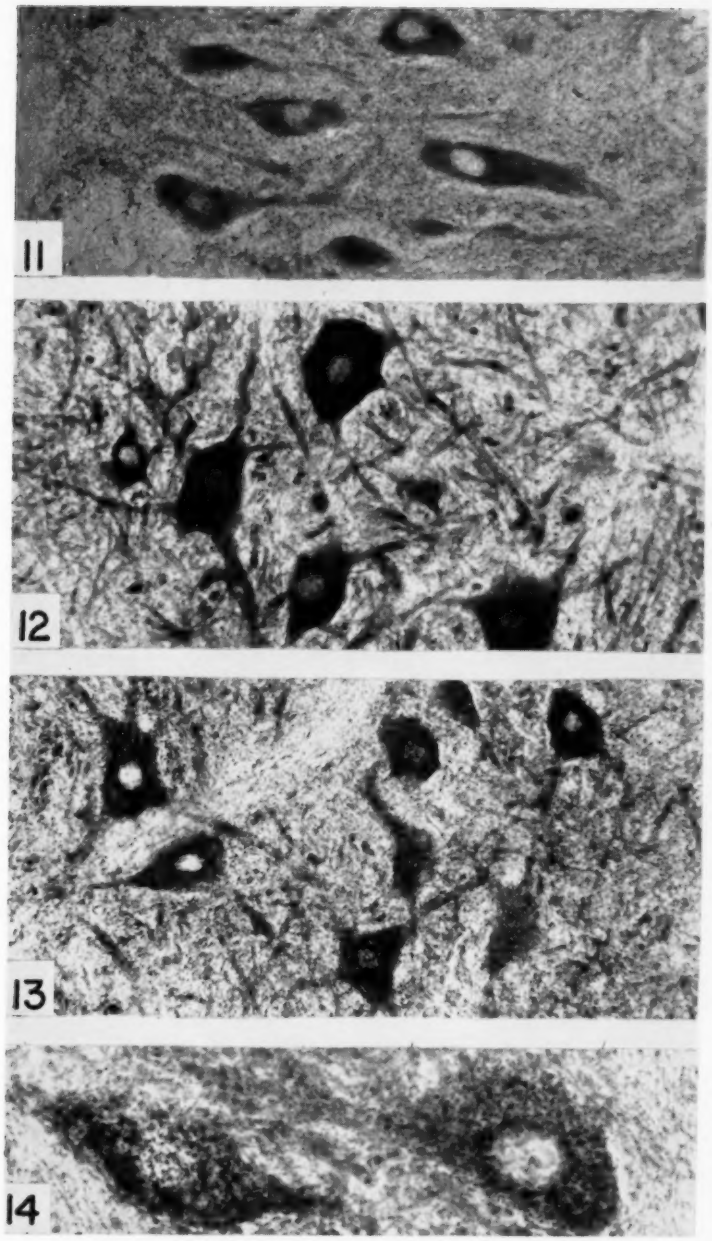


FIG. 11.—Fresh spinal cord, Nitro BT. A diffuse cytoplasmic reaction is present in the neurons of the anterolateral group. Dendritic processes (neuropil) are also visible. ×200.

FIG. 12.—Comparable area of spinal cord tissue as shown in Fig. 11. Fixed in 2% calcium–formalin previous to staining, Nitro BT. Note the increase in the intensity of the diaphorase reaction following the use of this fixative as compared with fresh, unfixed tissue. ×280.

FIG. 13.—Section of fixed (chloral-hydrate–formalin) spinal cord, Nitro BT. With this fixative, both diffuse and granular diaphorase localization is present in the cytoplasm. Neuropil is also positive. ×280.

FIG. 14.—Spinal cord fixed in 2% calcium–formalin and stained for diaphorase by the MTT method. Granular deposits of formazan are present in the cytoplasm of two anterior horn cells. ×645.

VOL.
5
1959/6

technique as used by us on nerve tissue not only produces an excellent visualization of tissue structure, but also provides useful information concerning metabolic activity.

CONCLUSIONS AND SUMMARY

1. The localization of DPN-diaphorase activity in neurons of the dorsal ganglia and spinal cord of the rat has been investigated histochemically by utilizing the coenzyme DPNH and either Nitro BT or MTT in the substrate. With this method, variable degrees of diaphorase activity are noted among the different cells of the dorsal ganglia. In the spinal cord, ventral horn cells located only in the ventromedial and ventrolateral regions are stained.

2. With MTT sites of diaphorase activity appeared as dot-like deposits of formazan which suggests intramitochondrial localization. The use of Nitro BT apparently demonstrates both intra- and extramitochondrial oxidative pathways by producing granular deposits of pigment as well as a strong diffuse formazan staining in the cytoplasm.

3. Brief fixation of dorsal ganglia and spinal cord segments in either chloral-hydrate–formalin or 2% calcium–formalin prior to the histochemical demonstration of DPN-diaphorase, not only succeeds in preserving enzyme activity, but actually causes an intensification of the reaction without any significant alteration in localization. Cold acetone causes a decrease in diaphorase activity.

4. Although extramitochondrial diaphorase activity is supposedly localized in microsomes which are rich in ribonucleic acid, preincubation of both fresh and fixed tissue sections in ribonuclease causes no appreciable decrease in the intensity of the reaction obtained with Nitro BT. The careful control of pH and substrate molarity in the incubating medium and the failure to demonstrate sulphydryl groups in tissue sections adds further support to the validity of our results.

Acknowledgements—The author is indebted to Dr. A. M. SELIGMAN for a gift of Nitro BT and Dr. A. G. EVERSON PEARSE who kindly provided a sample of MTT. The technical assistance of Mrs. BERTA POVENMIRE is gratefully acknowledged. Mr. EDWARD WEBSTER was responsible for the photomicrographs.

REFERENCES

BAKER J. R., HEW H. and FISHMAN W. H. (1958) *J. Histochem. Cytochem.* **6,** 244.
BARRNETT R. J. and SELIGMAN A. M. (1954) *J. nat. Cancer Inst.* **14,** 769.
ERNSTER L. (1956) *Exp. Cell Res.* **10,** 704.
ERNSTER L., JALLING O., LÖW H. and LINDBERG O. (1955) *Exp. Cell Res.* Suppl. **3,** 124.
ERNSTER L. and LÖW H. (1955) *Exp. Cell Res.* Suppl. **3,** 133.
FARBER E., STERNBERG W. H. and DUNLAP C. E. (1956) *J. Histochem. Cytochem.* **4,** 284.
FISHMAN W. H. and BAKER J. R. (1956) *J. Histochem. Cytochem.* **4,** 570.
GIACOBINI E. (1957*a*) *J. Neurochem.* **1,** 234.
GIACOBINI E. (1957*b*) *Acta physiol. scand.* **42,** Suppl. 145, 49.
GIACOBINI E. and HOLMSTEDT B. (1958) *Acta physiol. scand.* **42,** 12.
HESS R., SCARPELLI D. G. and PEARSE A. G. E. (1958) *J. biophys. biochem. Cytol.* **4,** 753.
NACHLAS M. M., WALKER D. G. and SELIGMAN A. M. (1958) *J. biophys. biochem. Cytol.* **4,** 29.
PEARSE A. G. E. Personal communication.
SELIGMAN A. M. and RUTENBURG A. M. (1951) *Science* **113,** 317.
SCARPELLI D. G., HESS R. and PEARSE A. G. E. (1958) *J. biophys. biochem. Cytol.* **4,** 747.
TSOU K. C., CHENG C. S., NACHLAS M. M. and SELIGMAN A. M. (1956) *J. Amer. Chem. Soc.* **78,** 6139.
ZIMMERMANN H. and PEARSE A. G. E. (1959) *J. Histochem. Cytochem.* **7,** 271.

Journal of Neurochemistry, 1960, Vol. 5, pp. 354–358. Pergamon Press Ltd. Printed in Northern Ireland

EFFECT OF MORPHINE ON THE UPTAKE AND OXIDATION OF GLUCOSE BY RABBIT BRAIN*

ROBERT SIMINOFF†‡ and PAUL R. SAUNDERS
Department of Pharmacology, School of Medicine
University of Southern California, Los Angeles, Calif.

(*Received* 7 *December* 1959)

VOL.
5
1959/6

ACHOR and GEILING (1956) found that morphine inhibited the uptake of ^{14}C into the brain of rats following the intravenous injection of [^{14}C] glucose. This effect was antagonized partially by nalorphine, and the data were interpreted as evidence that morphine depressed the uptake of glucose or its metabolites into the brain. We have investigated the effect of morphine upon the glucose concentration in the brain of rabbits *in vivo* and upon glucose uptake by brain slices *in vitro*. The latter experiments have been carried out both with normal and with potassium ion-stimulated slices; it has been shown (WEBB and ELLIOTT, 1951; MCILWAIN, 1953; LINDAN, QUASTEL and SVED, 1957; TAKAGAKI and TSUKADO, 1957, 1958) that high concentrations of K^+ increased the oxygen consumption and glucose uptake of brain slices, and we thought it possible that morphine might affect the metabolism of stimulated tissue to a different extent than was the case with unstimulated brain slices. It has been shown, for example, that the oxygen consumption of K^+-stimulated (GHOSH and QUASTEL, 1954; LINDAN *et al.*, 1957) and electrically-stimulated (MCILWAIN, 1953) brain slices is more readily inhibited by a number of centrally-acting drugs than is the unstimulated metabolism.

We have also studied the oxidation of [^{14}C] glucose by normal and K^+-stimulated slices prepared from the brains of rabbits previously given morphine in doses sufficient markedly to depress respiration.

METHODS

Preparation of blood filtrate. Blood was obtained by cardiac puncture just before the animal was stunned by a blow to the back of the neck. One ml of the oxalated blood was diluted with 4 ml of water and 10 ml of 5% (w/v) $ZnSO_4$ solution; then 10 ml of 0·3 N-$Ba(OH)_2$ solution were added with constant stirring, and the mixture was filtered.

Preparation of brain filtrate. The brain was rapidly exposed with bone forceps immediately after the animal had been stunned. The brain was scooped out and dropped into liquid nitrogen. The time interval between stunning the animal and dropping the brain into liquid nitrogen was 30–70 sec, although the brain was placed in liquid nitrogen within 4 sec after separation from its blood supply. The frozen tissue was rapidly weighed and transferred to a cold Waring microblendor. 5% $ZnSO_4$ solution was added (10 ml/g tissue) and the mixture blended for 1 min after which water (4 ml/g tissue) and 0·3 N-$Ba(OH)_2$ (10 ml/g tissue) solution were added and the mixture was again blended for 1 min and filtered.

* This investigation was supported (in part) by a PHS research grant (B-1802) from the National Institute of Neurological Diseases and Blindness, Public Health Service.

† This paper represents a portion of the research conducted by ROBERT SIMINOFF in partial fulfillment of requirements for the degree of Doctor of Philosophy in pharmacology.

‡ Present address: Department of Anatomy, University of California, School of Medicine, Los Angeles Calif.

Determination of glucose. Glucose was determined with the enzyme glucose oxidase (HUGGETT and NIXON, 1957). 2·5 ml of 'Glucostat' reagent (Worthington Biochemical Corp., Freehold, N.J.) were used with 2·0 ml of brain filtrate or 1·0 ml of blood filtrate plus 1·0 ml of water.

Effect of morphine upon uptake of glucose in vivo. The rabbit was tied to an animal board and after about 30 min morphine was administered (20 mg of morphine base/kg) either intraperitoneally or intravenously. Blood was taken by cardiac puncture after the maximal depression of respiratory rate (to about 10 per cent of the pre-injection value) had occurred (SIMINOFF and SAUNDERS, 1958) and the rabbit was then killed, the brain was removed, and glucose determined as described above.

Effect of morphine upon the uptake of glucose by various portions of brain in vitro. Uptake of glucose was measured in slices of rabbit brain incubated in a Dubnoff apparatus. Morphine was administered intravenously to the rabbit as described above; the animal was stunned, the entire brain was removed, and the cerebellum and brain stem were separated from the cerebral cortex. Thin free hand slices were prepared from cerebral cortex by frontal cuts such that both grey and white matter were included, from brain stem by transverse cuts at the level of the medulla and pons, and from cerebellum by saggital section. Slices were transferred to a small square of aluminium and weighed. The square was pressed to the bottom of a 50 ml beaker, and the aluminium was peeled away and reweighed to give the weight of tissue used. The beaker was placed in a Dubnoff shaker at 37° and 5 ml of bicarbonate-saline medium (MCILWAIN, 1955) containing 6 mM-glucose were added; the atmosphere was 95% O_2–5% CO_2. 0·2 ml of medium was removed at 1, 2, and 4 hr and diluted with 5 ml of water, and glucose determined in a 2 ml aliquot. Duplicate control vessels were run simultaneously in the absence of tissue, and the difference between the amount of glucose in the control and experimental vessels represented the glucose uptake. In order to measure the effect of morphine upon uptake of glucose by stimulated brain slices, KCl solution was added to the medium initially to give a final K^+ concentration of 44 mM. The glucose uptake of both normal and K^+-stimulated slices remained essentially constant over the 4 hr incubation period.

Effect of morphine upon uptake and oxidation of glucose by cerebral cortex slices. Phosphate–saline medium (LINDAN *et al.*, 1957) containing 6 mM-glucose was used. Uniformly-labelled [^{14}C] glucose (Nuclear-Chicago) was incorporated into the medium (200,000 to 400,000 counts/min per flask). The radioactivity of the glucose was determined by combustion by the method of VAN SLYKE *et al.* (1951) and counting as $BaCO_3$. Animals were injected intravenously with morphine as described above (20 mg/kg) and slices of cerebral cortex (0·35 mm in thickness) were prepared with a Stadie-Riggs microtome. The cerebral cortex was chilled in cold medium, cut into approximately 1 cm squares, and one slice was cut from the surface in such a way that only grey matter was included. The slice was transferred on a cover slip to the Warburg vessel (approximately 100 mg of tissue per vessel). To half of the vessels was added 0·1 ml of 3 M-KCl to give a final K^+ concentration of 100 mM. 3 ml of medium were added to each vessel, and 0·1 ml of N-H_2SO_4 to the side-arm. Experiments were run at 37° in air in a Warburg apparatus. The experiments were terminated after 2 hr by tipping in acid from the side-arm, and a 0·2 ml portion of the medium was taken 20 min later for the determination of glucose. For the determination of [^{14}C]CO_2 the filter paper and KOH solution in the centre well were transferred to a tube, water was added, and the filter paper removed after 30 min. 1 ml of 3·5% (w/v) $NaCO_3$ solution was added, and the carbonate was precipitated with saturated $BaCl_2$ solution; the $BaCO_3$ was collected on a filter paper as described by ARONOFF (1956) and counted.

A blank vessel containing no tissue was set up as described above. A 0·2 ml portion was removed at the end of the experiment for glucose determination; $BaCO_3$ was prepared from the contents of the centre well and no radioactive CO_2 was found.

The amount of [^{14}C] glucose oxidized to CO_2 was calculated from the fraction of added counts recovered in the $BaCO_3$.

RESULTS

There were no significant differences in the blood-brain glucose concentration ratios of normal and morphinized rabbits (Table 1). In some of the experiments, a blood sample was taken from the heart immediately after the brain had been removed, rather than before. The glucose content of such post-mortem samples was about 25 per cent higher, but the blood-brain ratios of control and morphinized animals were again not significantly different.

TABLE 1.—CONCENTRATION OF GLUCOSE IN BLOOD AND BRAIN AFTER ADMINISTRATION OF MORPHINE TO RABBITS

Data from animals given the drug intraperitoneally or intravenously were pooled

Dose of morphine (mg/kg)	Mean conc. of glucose		Ratio $\frac{\text{(blood glucose)*}}{\text{(brain glucose)}}$
	Blood (mg/100 ml)	Brain (mg/100 g wet wt)	
0	132	44	3·2 ± 0·9 (5)†
20	123	46	2·8 ± 0·7 (8)

* Mean values ± S.D.
† Number of animals given in parentheses.

TABLE 2.—EFFECT OF MORPHINE ON UPTAKE OF GLUCOSE BY NORMAL AND K^+-STIMULATED RABBIT BRAIN SLICES

Tissue	Experimental conditions	Glucose uptake* (μmole/g wet wt per hr)	
		Control	Morphine†
Cerebral cortex	Normal	18 ± 1‡	17 ± 1
	Stimulated	32 ± 5	33 ± 5
Brain stem	Normal	15 ± 4	15 ± 6
	Stimulated	20 ± 9	21 ± 9
Cerebellum	Normal	24 ± 10	20 ± 10
	Stimulated	36 ± 11	36 ± 11

* Each value for glucose uptake represents the mean of the values from 18 individual vessels containing tissue from a total of 3 rabbits. The tissue from each rabbit was divided equally into vessels containing 6·2 mM-K^+ (unstimulated slices) and 44 mM-K^+ (stimulated slices).
† 20 mg of morphine/kg was administered intravenously, except in one experiment with cortex in which 40 mg/kg was given.
‡ Standard deviation.

TABLE 3.—EFFECT OF MORPHINE UPON UPTAKE OF GLUCOSE AND UPON $[^{14}C]CO_2$ PRODUCTION FROM $[^{14}C]$ GLUCOSE BY NORMAL AND K^+-STIMULATED RABBIT CEREBRAL CORTEX SLICES

Metabolic change	Experimental conditions	Rate of metabolism* (μmole/g wet wt per hr)	
		Control	Morphine†
Glucose uptake	Normal	16 ± 3‡	15 ± 2
	Stimulated§	26 ± 5	22 ± 4
$[^{14}C]$ glucose oxidized to $[^{14}C]CO_2$	Normal	4·1 ± 0·3	4·6 ± 0·5
	Stimulated	6·3 ± 0·7	6·6 ± 0·9

* Each value represents the mean of the values from 9 vessels containing tissue from a total of 3 rabbits.
† 20 mg of morphine/kg was administered intravenously prior to preparation of slices.
‡ Standard deviation.
§ 100 mM-K^+ present in vessels.

VOL. 5 1959/6

Morphine had no effect on the uptake of glucose *in vitro* by either unstimulated or K^+-stimulated slices of cerebral cortex, brain stem, or cerebellum (Table 2).

In the studies of the uptake of glucose and of the oxidation of [^{14}C] glucose to [^{14}C]CO_2 by unstimulated and K^+-stimulated cerebral cortex slices (Table 3), to eliminate the possibility of loss of morphine from the tissue to the medium, 2·0 μg of morphine/ml were added to the medium; this concentration is approximately the peak value in the brain after the administration of 20 mg/kg of morphine intraperitoneally (SIMINOFF and SAUNDERS, 1958). The data show that morphine did not inhibit the uptake or oxidation of glucose by either unstimulated or K^+-stimulated slices. The oxygen consumption (mean values of 46 and 58 μmoles/g wet wt per hr for normal and K^+-stimulated strips respectively) was also not affected by morphine; BELL (1958) also found no effect of low concentrations of the drug upon the oxygen consumption of brain slices.

DISCUSSION

ACHOR and GEILING (1956) found that morphine inhibited the uptake of glucose or its derivatives by the brain *in vivo*. If uptake of glucose itself were depressed, one would expect its concentration in the brain to decrease rapidly after the administration of the drug because glucose is the primary substrate for this tissue (ELLIOTT, 1955). It is clear (Table 1) that no such decrease occurred in our experiments *in vivo*. It is also apparent (Tables 2 and 3) that the drug had no effect upon the uptake of glucose by brain slices *in vitro*. It might be argued that morphine inhibits the oxidation of glucose as well as its uptake, in which case the concentration in the brain would not necessarily decrease; however, no such inhibition of oxidation of glucose occurred (Table 3).

High concentrations of K^+ stimulated the metabolism of brain slices, as has been shown previously (references given in Introduction). As with unstimulated slices, however, morphine had no effect upon the increased glucose uptake, oxygen consumption, or oxidation of [^{14}C] glucose in K^+-stimulated slices.

A number of workers have shown that morphine can exert effects upon various phases of brain metabolism (QUASTEL and WHEATLEY, 1932; SEEVERS and SHIDEMAN, 1941; WATTS, 1949; WANG and BAIN, 1953; WOLPERT *et al.*, 1956). In all instances, however, the concentration of drug required to produce the effect was 100 or more times greater than that found in the brains of animals administered effective but non-fatal doses of the drug; e.g. MILLER and ELLIOTT (1955) found a concentration of approximately 0·001–0·002 mM-morphine in rat brain after a dose of 5 mg/kg, and SIMINOFF and SAUNDERS (1958) found 0·007 mM-morphine in rabbit brain after a dose of 20 mg/kg. The work reported here in which normal pharmacological doses of the drug were used indicated an absence of any effect upon glucose uptake or oxidation by either normal or K^+-stimulated slices. BELL (1958) has investigated the effect of morphine upon oxygen consumption and lactic acid formation in normal and electrically-stimulated rat cerebral cortex and diencephalon *in vitro*, and found that the increased metabolism associated with electrical stimulation was inhibited by concentrations of morphine which were without effect upon unstimulated tissue. The concentration required to depress the increased metabolism significantly, however, was 1 mM, which is about 1000 times that found in rat cerebral hemispheres following administration of the drug in effective doses (MILLER and ELLIOTT, 1955). It does not appear likely, therefore, that the analgesic effect of morphine can be explained on the

basis of an effect upon the metabolism of glucose, unless (as pointed out by BELL, 1958) localization of the drug in specific areas of the brain occurs.

SUMMARY

Morphine was administered to rabbits in doses producing approximately 90 per cent depression in respiratory rate. The blood/brain ratio of glucose concentrations in these animals did not differ significantly from those in normal animals. Furthermore, the glucose uptake and the production of $[^{14}C]CO_2$ from added [^{14}C] glucose by unstimulated and K^+-stimulated brain slices prepared from normal and morphinized animals also did not differ significantly. The depressant effects of morphine can therefore not be explained on the basis of an inhibition of the metabolism of glucose by the brain, unless specific areas of the brain respond differently from the brain as a whole.

REFERENCES

ACHOR L. B. and GEILING E. M. K. (1956) *Arch. int. Pharmacodyn.* **105,** 313.
ARONOFF S. (1956) *Techniques of Radiobiochemistry* p. 48. Iowa State College Press, Ames.
BELL J. L. (1958) *J. Neurochem.* **2,** 265.
ELLIOTT K. A. C. (1955) In *Neurochemistry. The Chemical Dynamics of Brain and Nerve* (Edited by ELLIOTT K. A. C., PAGE I. H. and QUASTEL J. H.) p. 61. Thomas, Springfield, Ill.
GHOSH J. M. and QUASTEL J. H. (1954) *Nature, Lond.* **174,** 28.
HUGGETT A. St. G. and NIXON D. A. (1957) *Lancet* ii, 368.
LINDAN O., QUASTEL J. H. and SVED S. (1957) *Canad. J. Biochem. Physiol.* **35,** 1135.
MCILWAIN H. (1953) *Biochem. J.* **53,** 403.
MCILWAIN H. (1955) *Biochemistry and the Central Nervous System* p. 39. Little, Brown, Boston.
MILLER J. W. and ELLIOTT H. W. (1955) *J. Pharmacol.* **113,** 283.
QUASTEL J. H. and WHEATLEY A. H. M. (1932) *Proc. Roy. Soc.* B **112,** 60.
SEEVERS M. H. and SHIDEMAN F. E. (1941) *J. Pharmacol.* **71,** 373.
SIMINOFF R. and SAUNDERS P. R. (1958) *J. Pharmacol.* **124,** 252.
TAKAGAKI G. and TSUKADA Y. (1957/58) *J. Neurochem.* **2,** 21.
VAN SLYKE D. D., PLAZIN J. and WEISIGER J. R. (1951) *J. biol. Chem.* **191,** 299.
WANG R. I. H. and BAIN J. A. (1953) *J. Pharmacol.* **108,** 354.
WATTS D. T. (1949) *J. Pharmacol.* **95,** 117.
WEBB J. L. and ELLIOTT K. A. C. (1951) *J. Pharmacol.* **103,** 24.
WOLPERT A., TRUITT E. B., BELL F. K. and KRANTZ J. C. (1956) *J. Pharmacol.* **117,** 358.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, pp. 359–369. Pergamon Press Ltd. Printed in Northern Ireland

METABOLIC DEFECTS IN A CONVULSIVE STRAIN OF MOUSE

H. NARUSE, M. KATO, M. KUROKAWA, R. HABA and T. YABE
Department of Neuropsychiatry, Faculty of Medicine, University of Tokyo, and Neuropsychiatric Research Institute, Seiwa Mental Hospital, Tokyo

(*Received* 11 *November* 1959)

EVIDENCE has accumulated that there are rapid changes in certain brain constituents during convulsive activity. RICHTER and DAWSON (1948) have shown that the brain ammonia level is markedly increased by direct electrical stimulation of the rat brain or by procedures which increase cerebral irritability. Brief electrical stimulation of the rat brain also caused a rapid fall of acetylcholine (RICHTER and CROSSLAND, 1949) and creatine phosphate (DAWSON and RICHTER, 1950) in the preconvulsive period. More recently, a defect was reported in human epileptogenic 'focal' tissues, which show a reduced resynthesis of glutamic acid. Tissues from methionine sulphoximine-treated animals have also shown a similar pattern of defective metabolism (TOWER, 1955).

A causal relationship between the convulsive activity and the changes in brain constituents would be further demonstrated, if convulsions could be produced in animals by a more physiological method of stimulation. A more or less persistent defect of metabolism which predisposes to convulsive disorders would be of much interest.

The present paper describes a special strain of mouse in which convulsions can be induced by means of 'postural' stimulation, which is without effect when applied to ordinary strains. The form of stimulation that is used is an alteration of posture, unbalancing the mouse. The biochemical defects studied in this strain concern certain aspects of the metabolism of free amino acids, ammonia and acetylcholine in the brain.

METHODS

Experimental animals. The pure convulsive strain of mouse used in these experiments was discovered in 1954 by IMAIZUMI and named the *ep* strain. The convulsive characteristic appears to be hereditary as a Mendelian dominant. Exogenous factors known to cause convulsive disorders, such as infections and dietary deficiencies have been specifically eliminated (IMAIZUMI *et al.*, 1959). As controls, mice of *gpc* and *dd* strains were used. All animals were bred in our laboratory and received the usual diet with some greens

Convulsions of ep *strain.* Successive and alternating movements causing loss of postural equilibrium of adult mice were the effective stimuli which induced convulsions in the strain. Of the different stimuli which were used, the simplest and most effective was to throw the mouse up in the air 15–20 cm several times. This inevitably caused muscular activity which complicated the biochemical study of the animal. With this method, the limbs were stimulated when they came into contact with the plate which supported the mouse. But this afferent stimulation was not likely to have induced the convulsions, since vertical swinging of a mouse with the limbs loosely fixed to a plate was also effective in inducing convulsions. Similarly, to-and-fro horizontal swinging, pendulum movement and alternating rotation also proved effective. Continuous rotation in one direction never caused convulsions, showing that the alternating character of stimulation was essential.

In the majority of mice, 'seesaw stimulation' was used routinely as this was the most easily standardized procedure and the least muscular movements were observed during this form of stimulation. The mouse was placed with its corporeal axis in the central axis of a plate; the plate was then subjected to a seesaw motion about the axis, at an angle of inclination of 15°, with a frequency of 25 cycles per 10 sec (Fig. 1).

The minimal period of 'seesawing' needed to provoke seizures varied with different mice from 5 to 35 sec. It appeared however that the period of threshold stimulation was almost constant for a particular mouse and this greatly facilitated the quantitative description of stimuli. The minimum

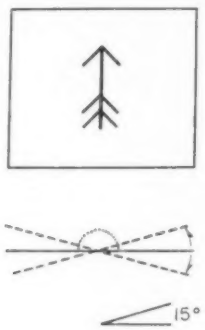


FIG. 1.—'Seesaw stimulation'. The mouse is placed with its long axis in the central axis of a plate; the plate is subjected to a seesaw motion about the axis, at an angle of inclination of 15°, with a frequency of 25 cycles per 10 sec.

period required to induce convulsions by throwing the animal up in the air was usually shorter than with seesaw stimulation, being from 2 to 12 msec.

Experimental arrangements. Unless otherwise stated, adult mice of both sexes, at least 12 weeks old and weighing 20 to 30 g were used.

Littermates of *ep* mice usually numbering 8–10 were divided into two groups; in the 'non-stimulated' control, seesaw stimulation was never given, while in the 'stimulated' series it was given regularly once a week from the beginning of the 7th week after birth. The number of stimulations applied to each mouse before being used in an experiment was 8–10, with a minimum of 5. Mice in which convulsive seizures were brought on by seesaw stimulation applied for 8–16 sec were mainly used for the study of acetylcholine; another series, which needed stimulation for 24–32 sec, was used for the study of ammonia and amino acids of the brain.

For determining resting levels of acetylcholine, ammonia and amino acids, the mouse was left in a wooden box insulated from sensory stimuli for more than 30 min prior to fixation in liquid nitrogen. At the beginnning of the experiment, a trap was released and the mouse dropped instantaneously into liquid nitrogen. In some animals, seesaw stimulation was given for 8 or 20 sec to obtain preconvulsive values. Values in the refractory period were those obtained in animals fixed between 10 and 15 min after cessation of convulsive movements. Other conditions are given in the respective Tables.

In the experiments indicated, mice were placed in a desiccator in which the air was replaced by 99·98% nitrogen. After transient excitement due to anoxia, the mouse lost its postural control in about 30 sec and went into coma after 40 sec. Convulsions were never elicited during anoxia lasting up to 120 sec. Determinations were carried out in brains fixed between 40 and 50 sec of anoxia.

Convulsions were brought on in control strains by intramuscular injection of pentamethylenetetrazole (80 mg/kg) or 3-methyl-3-ethylglutarimide (37 mg/kg). The intramuscular route for injection was preferred to others because of the slower onset of convulsions. Convulsive seizures occurred 3–5 min after injection, but the form of seizures differed with the two convulsants, being atypical with 3-methyl-3-ethylglutarimide. Determinations were carried out in mice fixed either at the preconvulsive stage (1–2 min after injection) or during the initial stage of convulsions.

Determinations. The frozen brain was rapidly dissected out without allowing it to thaw, and finely powdered in a cooled steel crusher. The term 'brain' refers to the whole brain without the

VOL. 5 1959/6

cerebellum. In ammonia and amino acid determinations, respectively, the powder (150–120 mg) was dropped into a previously weighed centrifuge tube containing 4 ml of 10% (w/v) or 4·5 ml of 5% (w/v) trichloroacetic acid (TCA) at 0°, reweighed, finely ground and centrifuged at 12,500 rev/min for 5 min.

The free ammonia content of the brain was determined in 0·5 ml of supernatant solution (10% TCA) by CONWAY's (1947) micro-diffusion method. The amount of ammonia due to the hydrolysis of glutamine under the conditions used was estimated and a correction was made accordingly (RICHTER and DAWSON, 1948). The glutamine content was determined by the method of HARRIS (1943) as described by RICHTER and DAWSON (1948).

Glutamine, glutamic and γ-aminobutyric acids were separated and determined in 4 ml of supernatant solution (5% TCA) by the method of BERL and WAELSCH (1958), based on ion-exchange chromatography followed by the appropriate hydroxamate determination. Slight modifications were made in the volumes of elution buffers, and the determination of glutathione described in the original method was not carried out.

Total acetylcholine was extracted with buffered saline, as described by RICHTER and CROSSLAND (1949). The powdered brain was stirred at 0° into a mixture of 1 ml acetate buffer and 3 ml eserinized (250 μg eserine sulphate/ml) amphibian Ringer-solution at pH 4. The wet weight of tissue was determined by differential weighing, the mixture was boiled for 2 min, allowed to cool and centrifuged for 10 min at 3,000 rev/min. The residue was re-extracted with 1 ml eserinized acidified amphibian Ringer-solution and the combined supernatant fluids were used for assay. The 'free' acetylcholine was extracted from the powdered brain with eserinized (200 μg eserine sulphate/ml) amphibian Ringer-solution at pH 7. The acetylcholine assay was carried out using the eserinized rectus muscle of the frog. The sample solution was brought to pH 7 and diluted with amphibian Ringer-solution so that each ml contained the extract from 20 mg of brain. The solution was assayed against known amounts of acetylcholine chloride to which the brain extract freed from acetylcholine by hot alkali treatment was added. This was reported to control for the non-specific effect of tissue extracts on the contraction of the rectus (FELDBERG, 1945; RICHTER and CROSSLAND, 1949). Extraction and assay of acetylcholine were carried out at a room temperature below 10°, or in a cooled room when necessary. The 'bound' acetylcholine was calculated as a difference between the total and 'free' acetylcholine.

The rate of acetylcholine resynthesis was measured in isolated brain tissues. Animals were decapitated by a single sharp cut with scissors, and slices 0·3–0·4 mm in thickness were cut from the cerebral cortex with a blade. Approximately 50 mg of tissue were placed in the conventional manometric flask containing 2·8 ml of experimental saline. The saline contained 127 mM-NaCl, 5·1 mM-KCl, 1·3 mM-KH_2PO_4, 1·3 mM-$MgSO_4$, 2·7 mM-$CaCl_2$, 10 mM-sodium phosphate buffer at pH 7·4 and 10 mM-glucose, and was gassed with oxygen. In the experiments indicated, the concentration of KCl was raised to 33 mM; this caused an increase in osmotic pressure which was not corrected. The manometers were placed in the thermostat within 30 min of the death of the animal and equilibrated for 15 min. At zero time, 0·2 ml eserinized phosphate–saline were added from the side-arm to give a final concentration of 400 μg eserine sulphate/ml. Oxygen uptake was then followed manometrically for 60 min at 38·0°. After incubation, the slice was quickly taken out of the flask, rinsed with, and finely ground in, eserinized amphibian Ringer-solution at pH 4, and centrifuged. The acetylcholine of the supernatant solution was then assayed. The amount of 'free' acetylcholine was determined in portions of the incubation media. Corrections were made for potassium concentration with potassium-free amphibian Ringer-solution so that the final assay sample contained the same amount of potassium ions as did the amphibian Ringer-solution. All quantities of acetylcholine are given in terms of the chloride.

For measurement of brain cholinesterase activity, the cerebral cortex was finely ground in bicarbonate–saline to give an 0·5% (w/v) brain suspension. The saline, containing 124 mM-NaCl, 2·5 mM-KCl, 1·8 mM-$CaCl_2$ and 25·0 mM-bicarbonate buffer at pH 7·0 was gassed with 5% CO_2–95% N_2. Two ml of the suspension were then pipetted into a conventional manometric flask and equilibrated for 10 min prior to addition from a side-arm of 0·2 ml of 1% (w/v) acetylcholine chloride solution in bicarbonate–saline. The final substrate concentration was 5×10^{-3} M, or 2 mg acetylcholine for 10 mg of tissue. Under these conditions, carbon dioxide evolution due to acid formed by the hydrolysis of acetylcholine was found to bear a linear relationship to time during the incubation period of 60 min at 37·5°.

RESULTS

A typical seizure begins with prodromal symptoms of very short duration, most commonly sharp squeaks and catatonic posture. During the catatonic stage the animal stands perfectly still with its tail erect, and the postural control is still well maintained. One to three seconds later, there is a characteristic 'breast-stroke swimming' motion or 'running fit' followed by convulsions, and the postural control is lost. Convulsions, at first clonic in nature, start in the hind limbs, but rapidly pass into a global tonus-clonus. During the generalized convulsive stage, massive salivation and urinary incontinence are usually seen. Cessation of convulsive activity is followed by a stage of post-ictal depression, characterized by inertness and stupor, or less commonly by excitement with extreme irritability. In 22 animals, the time from the first squeak to the complete cessation of convulsive movement was 16 $\pm$ 3 (S.D.) sec.

There is a definite refractory period before the second stimulus is effective, but its duration showed fairly wide individual variation. Of 20 animals tested, 6 had a refractory period of 30 min, while 6 would not convulse even after 40 min.

Convulsive seizures with postural stimulation could never be induced in animals younger than 7 weeks. Reproductive function in the strain was generally attained in the course of the 7th week after birth, suggesting the influence of hormonal factors in the occurrence of seizures.

From the 7th to 10th week, seizures generally took an abortive form, most commonly squeaks and catatonic posture, occasionally simple muscular twitchings. Stimulation was usually given once a week from the beginning of the 7th week, and complete convulsions could then be produced in the course of the 10th or 11th week, i.e. after 3 or 4 stimulations. If, however, the animals were first stimulated after they were older than 11 weeks, some responded with typical seizures, showing that previous stimulation was not absolutely essential for the genesis of convulsions; a particular neurological constitution at a definite stage of development might be the critical factor. Convulsions were produced in all animals of both sexes. Convulsions in this strain did not seem to decrease with age; e.g. they could be produced as easily in a mouse 60 weeks old, although the mean life of *ep* mice was approximately 30 weeks. Mice of the *ep* strain did not die from the convulsions: this is in sharp contrast to audiogenic strains, of which 30 per cent subjected to excessive acoustic stimulation were reported to have died after convulsions, apparently from respiratory paralysis (SUTER *et al.*, 1958).

The convulsive threshold to an applied convulsant was lower in the *ep* than in the *gpc* strain: the minimal dose of pentamethylenetetrazole effective to induce generalized convulsions after intramuscular injection was 40 mg/kg and 70 mg/kg in *ep* and in *gpc* mice respectively.

Histological examination of the mouse brain tissues by Nissl, haematoxylin-eosin and Benzidine-Pickworth staining techniques failed to show defects which could be considered as a morphological basis for, or a result of, convulsive disorder in *ep* mice.

Ammonia. The resting ammonia level of *ep* mice was about 40 per cent lower than that of controls, regardless of whether or not convulsions had been previously produced by seesaw stimulation (Table 1).

A marked rise in brain ammonia was induced in *ep* mice by applying seesaw stimulation, the level being 180, 250 and 470 per cent above the resting control with 8, 20 and 24 sec stimulations respectively. The muscular activity accompanying

VOL.
5
1959/6

. Seesaw stimulation applied to *ep* mice for 24 sec, the minimal period causing convulsions, resulted in a slight but not significant decrease in the glutamic acid level. It did not alter the glutamine and GABA contents (Table 2).

Acetylcholine. The acetylcholine level of the brain of *ep* mice was 40–60 per cent higher than that of the other strains. Further, it was shown that for the *ep* and the *gpc* strains the acetylcholine level was lower in immature mice and increased until about the 7th week after birth. For instance, values at the 4th, 5th, 6th and 7th week

TABLE 2.—GLUTAMINE, GLUTAMIC AND γ-AMINOBUTYRIC ACIDS IN ADULT MOUSE BRAIN

Mice were killed by immersion in liquid nitrogen. For details of conditions, see Experimental arrangements.

Strains	Conditions	No. of mice	Glutamine (μmoles/g)	Glutamic acid (μmoles/g)	γ-Amino-butyric acid (μmoles/g)
ep	Resting	4	3·0 ± 0·2	7·0 ± 0·7	3·3 ± 0·2
	Seesaw stim., 24 sec, convulsions	4	3·2 ± 0·3	6·2 ± 0·4	3·2 ± 0·2
gpc	Resting	4	4·5 ± 0·4	9·4 ± 0·7	2·4 ± 0·2
dd	Resting	3	4·3	10·1	2·1

were, for the *ep* strain, 2·09, 2·31, 2·54 and 2·66, and for the *gpc* strain, 1·47, 1·65, 1·94 and 1·95 μg/g.

Seesaw stimulation for 8 sec caused a fall in the acetylcholine content of *ep* mice. The fall was greater in the 'stimulated' series in which seesaw stimulation was given regularly once a week than in the 'non-stimulated' series. In the 'stimulated' series the fall amounted to 0·6 μg/g during the preconvulsive stage and to 1 μg/g after the onset of convulsions and this fall was maintained during the period of convulsions. In mice fixed in liquid nitrogen 60 sec after cessation of the convulsions the normal acetylcholine level was restored. In the 'non-stimulated' series a period of 8 or 40 sec seesaw stimulation produced no convulsions and the acetylcholine level fell by only 0·3–0·4 μg/g. When the seesaw stimulation was applied in the 'stimulated' series during the refractory period in which it produced no convulsions the acetylcholine level fell by only 0·2–0·3 μg/g (Table 3).

In the control strains, *gpc* and *dd*, seesaw stimulation for 8 sec caused no convulsions and no significant reduction in the acetylcholine level of the brain. In these animals the injection of pentamethylenetetrazole, however, produced convulsions and a pronounced reduction in the acetylcholine level which occurred even in the preconvulsive stage (Table 3).

Anoxic coma induced by pure nitrogen, which caused a rise in brain ammonia, did not affect the acetylcholine level of the brain (Table 3).

Since the changes in acetylcholine content of the brain were not paralleled by changes in 'free' acetylcholine, they must have occurred in the 'bound' fraction. This is evident when the values for 'bound' acetylcholine are calculated from the difference between total and 'free' acetylcholine (Table 3).

Respiration and acetylcholine resynthesis in isolated brain tissues. No significant

seesaw stimulation was unlikely to have caused the secondary rise of brain ammonia, although the blood ammonia level could not be determined under the present experimental conditions. In the control mice, the same stimulation did not cause any change in the brain ammonia; no change was observed even with stimulation lasting for as long as 240 sec.

The increase in brain ammonia of *ep* mice with postural stimulation closely paralleled that of control mice which were chemically convulsed. Mice injected with

TABLE I.—AMMONIA AND GLUTAMINE IN ADULT MOUSE BRAIN UNDER VARIOUS CONDITIONS

Gpc and *dd* strains were taken as controls. For details of treatment and description of 'stimulated' and 'non-stimulated' series, see Experimental arrangements. Mean values are given ± standard deviation.

Strains	Treatments and remarks	No. of mice	Ammonia (μmoles/g)	Glutamine (μmoles/g)
ep	Resting (*non-stimulated*)	6	0·13 ± 0·01	2·50 ± 0·21
	Resting (*stimulated*)	10	0·11 ± 0·03	2·44 ± 0·28
	Seesaw stim., 8 sec, preconvulsive	6	0·20 ± 0·03	2·33 ± 0·34
	Seesaw stim., 20 sec, preconvulsive	6	0·28 ± 0·06	2·46 ± 0·21
	Seesaw stim., 24–32 sec, convulsions	6	0·52 ± 0·06	2·53 ± 0·14
gpc	Resting	8	0·21 ± 0·02	3·26 ± 0·21
	Seesaw stim., 8 sec, no changes	6	0·18 ± 0·02	3·39 ± 0·34
	Seesaw stim., 240 sec. no changes	6	0·18 ± 0·02	3·18 ± 0·41
	PMT injection, preconvulsive	8	0·41 ± 0·06	
	PMT injection, convulsions	6	0·49 ± 0·06	3·42 ± 0·15
	MEG injection, preconvulsive	3	0·36	
	MEG injection, convulsions	4	0·53 ± 0·03	
	Anoxia in pure N_2, comatose	8	0·50 ± 0·07	
	Water injection, no changes	2	0·20	
dd	Resting	10	0·20 ± 0·02	3·67 ± 0·48
	Seesaw stim., 8 sec, no changes	4	0·18 ± 0·03	3·25 ± 0·41
	Seesaw stim., 240 sec, no changes	4	0·17 ± 0·02	3·25 ± 0·55
	PMT injection, preconvulsive	2	0·41	

PMT: Pentamethylenetetrazole (80 mg/kg), intramuscular.
MEG: Methylethylglutarimide (37 mg/kg), intramuscular.

pentamethylenetetrazole or with 3-methyl-3-ethylglutarimide showed markedly increased brain ammonia, both at the preconvulsive and at the convulsive stage, in accord with observations reported by RICHTER and DAWSON (1948) in rats given picrotoxin. In anoxic coma induced by pure nitrogen, brain ammonia was again markedly increased, in spite of the fact that convulsions were never elicited under these conditions (Table 1; cf. RICHTER and DAWSON, 1948).

Glutamine, glutamic acid and γ-aminobutyric acid. The results of glutamine, glutamic acid and γ-aminobutyric acid (GABA) determinations after their respective separation by ion-exchange chromatography are listed in Table 2. Both brain glutamine and glutamic acid of *ep* mice were found to be approximately 30 per cent lower than those of control mice. In contrast, the brain GABA content of *ep* mice was 40–50 per cent higher.

TABLE 3.—ACETYLCHOLINE IN ADULT MOUSE BRAIN IN DIFFERENT STATES OF ACTIVITY
For details of treatment, and for description of 'stimulated' and 'non-stimulated' *ep* mice, see Experimental arrangements. Values are given in terms of acetylcholine chloride.

Strains	Treatments and remarks	No. of mice	Total ACh (μg/g)	'Free' ACh (μg/g)	'Bound' ACh (μg/g)
ep: Stimulated	Resting	10	2·86 ± 0·10	0·72 ± 0·06	2·14
	Seesaw stim., 8 sec, preconvulsive	8	2·25 ± 0·28	0·63 ± 0·17	1·62
	Immediately after the first squeak	3	1·84	0·58	1·26
	Immediately after convulsions	4	1·75 ± 0·25	0·94 ± 0·20	0·81
	60 sec after convulsions	2	2·71		
	Seesaw stim., 40 sec, during refractory period	4	2·52 ± 0·19		
Non-stimulated	Resting	4	2·76 ± 0·10	0·70 ± 0·04	2·06
	Seesaw stim., 8 sec, no convulsions	4	2·42 ± 0·18	0·68 ± 0·12	1·74
	Seesaw stim., 40 sec, no convulsions	3	2·33		
gpc	Resting	5	1·95 ± 0·14	0·60 ± 0·10	1·35
	Seesaw stim., 8 sec, no changes	5	1·81 ± 0·13	0·57 ± 0·05	1·24
	PMT injection, preconvulsive	5	1·51 ± 0·14	0·54 ± 0·05	0·97
	PMT injection, convulsions	4	1·12 ± 0·17	0·49 ± 0·10	0·63
	Water injection, no changes	4	1·99 ± 0·07	0·61 ± 0·09	1·38
	Anoxia in pure N_2, comatose	3	1·82	0·57	1·25
dd	Resting	5	1·77 ± 0·10	0·55 ± 0·10	1·27
	Seesaw stim., 8 sec, no changes	4	1·66 ± 0·14	0·50 ± 0·03	1·16
	PMT injection, preconvulsive	6	1·35 ± 0·21	0·50 ± 0·10	0·85
	PMT injection, convulsions	3	1·07		
	Water injection, no changes	3	1·83	0·62	1·21
	Anoxia in pure N_2, comatose	5	1·88 ± 0·18	0·50 ± 0·10	1·38

PMT: Pentamethylenetetrazole (80 mg/kg), intramuscular.

differences were found in the respiratory rates of brain slices from the *ep* and the control *gpc* mice in phosphate media either with normal or with a high potassium concentration (Table 4).

With a potassium concentration of 6·5 mM in the test media, the rate of 'bound'

TABLE 4.—RESPIRATION AND ACETYLCHOLINE RESYNTHESIS IN CEREBRAL CORTEX OF ADULT MOUSE

Approximately 50 mg of grey matter slice were incubated at 38·0° in 3·0 ml of phosphate saline at pH 7·4 with 10 mM-glucose as substrate. Saline contained 400 μg/ml eserine sulphate.

Strains	K^+ concentration (mM)	No. of mice	'Free' ACh (μg/g)	'Bound' ACh (μg/g)	Respiration (μmoles/g/hr)
ep: Stimulated	6·5	3	2·50	12·83	104
	33	3	14·13	3·64	162
Non-stimulated	6·5	3	2·24	10·89	103
	33	3	14·19	3·30	157
gpc	6·5	5	1·71 ± 0·25	6·47 ± 0·30	100 ± 8
	33	5	9·69 ± 0·24	2·30 ± 0·91	157 ± 11

acetylcholine resynthesis in isolated brain tissues was found to be significantly higher in the *ep* than in the control strain (Table 4). The rate was slightly lower in the 'non-stimulated' than in the 'stimulated' *ep* mice, but this difference was not significant. With an increased potassium concentration of 33 mM, the increase in 'free' acetylcholine in the test media during incubation was larger in the *ep* than in the control mice; again no significant difference was found between the two series of *ep* mice.

Cholinesterase activity. Cholinesterase activity in the mouse brain 5–6 weeks after birth was shown to be approximately 60–70 per cent of the adult level reached in the 9th–10th week (Table 5). The esterase activity of *ep* mice tended to be higher than that of *gpc* mice, but the difference was not significant. A significant difference ($P < 0{\cdot}05$, by Student's '*t*' test) was noted in cholinesterase activity between the two series of *ep* mice; the activity in the 'stimulated' series was lower by 20 per cent when compared with that of the 'non-stimulated' series (Table 5).

DISCUSSION

The finding that postural stimulation of the *ep* mice increased the ammonia level

TABLE 5.—CHOLINESTERASE ACTIVITY OF THE CEREBRAL CORTEX OF MOUSE

The final concentration of acetylcholine chloride as substrate was 5×10^{-3} M for 10 mg of brain tissue ground in bicarbonate saline at pH 7·0.

Strains	Age (weeks)	No. of mice	ChE activity (μl CO_2/100 mg/hr)
ep: Non-stimulated	5–6	4	858 ± 42
	7–8	6	1029 ± 40
	9–10	3	1227
	12	4	1253 ± 49
Stimulated	12	4	1039 ± 42
gpc	5–6	4	730 ± 30
	7–8	6	915 ± 45
	9–10	3	1130
	12	4	1157 ± 65

VOL. 5 1959/6

of the brain in the preconvulsive and convulsive state is in accord with the view that the ammonia level in the brain is dependent on its functional activity. This was shown for convulsive activity induced by electrical and pharmacological stimulation (RICHTER and DAWSON, 1948; BENITEZ, PSCHEIDT and STONE, 1954), for the activity induced by conditioned reflexes (VLADIMIROVA, 1954; TSUKADA *et al.*, 1958) and for physical exercise (VRBA, 1954). Increases in brain ammonia can occur under conditions which do not lead to convulsions, as was shown in anoxic coma induced by pure nitrogen. VRBA and FOLBERGROVÁ (1959) have obtained evidence that the increased ammonia formation in the brain of rats subjected to continuous exercise is accompanied by an enhanced breakdown of brain protein as well as of nucleoprotein.

KOLOUŠEK, HORÁK and JIRÁČEK (1959) have reported a lower level of free ammonia-N, and a higher level of free amide-N in brain of rats which can be convulsed by acoustic stimuli. We also found that the brain ammonia level of *ep* mice was lower than that of our controls. In contrast to their observations, we found the free glutamine level low as well.

Values for glutamine and glutamic acid in brains of control mice (*gpc* and *dd* strains) agree reasonably well with those reported by previous workers (SCHWERIN, BESSMAN and WAELSCH, 1950). In contrast, values for GABA, 2·4 and 2·1 μmoles/g for *gpc* and *dd* mice respectively, were considerably lower than the value of 4 μmoles/g reported by ROBERTS, HARMAN and FRANKEL (1951), which was obtained using paper chromatographic separation followed by ninhydrin treatment. The difference may be attributable to the method of determination, since BERL and WAELSCH (1958) have obtained a mean value of 3·5 μmoles/g for GABA in the thalamus and hypothalamus of rat by the same method as we used; this value was the highest they found in the brain, but a still higher value (4·5 μmoles/g) was reported for the whole brain of the same species using paper chromatography and ninhydrin development (RUISSEAU *et al.*, 1957). Other data for GABA of mouse brain available for comparison appear to be still incomplete.

The reduced resynthesis of glutamic acid has been noted in incubated slices from human epiletogenic foci (TOWER, 1955). The low values of glutamine and glutamic acid found in the brain of *ep* mice might suggest the impairment of ammonia removal by the glutamine-synthesizing system, but there has been no direct evidence to support this idea. Also the reason for the raised level of GABA in *ep* mice remains to be further elucidated.

Since, in the present experiments, no changes occurred in the glutamine, glutamic acid and GABA levels of the brain in the *ep* mice convulsed by postural stimulation, the fall in glutamic acid and aspartic acid of the brain, which DAWSON (1953) observed in rats poisoned with fluoroacetate before the onset of, and during convulsions may be specifically associated with the fluoroacetate poisoning, or more likely, with the longer duration of the preconvulsive state.

The total acetylcholine level of the normal mouse brain, 1·95 $\pm$ 0·14 and 1·77 $\pm$ 0·10 (S.D.) μg/g for *gpc* and *dd* mice respectively, were somewhat lower than the value of 2·3 $\pm$ 0·22 (S.D.) μg/g found in the mouse brain by CROSSLAND and MERRICK (1954); this may partly be attributable to the species difference and partly to the different method of extraction used in the present experiment (cf. CROSSLAND, PAPPIUS and ELLIOTT, 1955).

The high level of acetylcholine in *ep* mice *in vivo* is in agreement with the enhanced

rate of acetylcholine resynthesis found in the cerebral cortex slices of the same strain. The high acetylcholine content was shown even in younger animals of the 'non-stimulated' series, suggesting that the secondary changes due to convulsions were not responsible.

RICHTER and CROSSLAND (1949) have shown a rapid fall (0·7 μg/g) and recovery of brain acetylcholine occurring in the course of the preconvulsive state produced by a brief application of electrical stimuli to the rat brain. A rapid recovery of acetylcholine prior to the onset of convulsions has not been demonstrated with our 'postural' or pharmacological stimulation, apparently because of the continuous nature of these stimuli.

In the present experiments, it is suggested that the conversion of a certain amount of 'bound' acetylcholine to the 'free' form, is closely related to the onset of convulsions, and that the conversion mechanism is partially inactivated in the 'non-stimulated' series and during the refractory period of the 'stimulated' series. A decrease of 0·7 μg/g acetylcholine was reported in rats during the depressed, preconvulsive stage of insulin hypoglycaemia (CROSSLAND, ELLIOTT and PAPPIUS, 1955); this was produced in a much longer time than with our 'postural' stimulation. It thus appears that a net decrease of acetylcholine, irrespective of the rate of breakdown, is involved in the onset of seizure.

In control strains, coma due to anoxia did not cause a significant change of acetylcholine, and convulsions were never produced under these conditions. However, this does not eliminate the possibility of transient changes in acetylcholine during the initial stage of anoxia when animals were excited (cf. RICHTER and CROSSLAND, 1949).

The 'free' acetylcholine extracted from the powdered tissue appeared to be largely an artifact produced during the extraction, since it showed a fairly constant value in different determinations (cf. ELLIOTT and HENDERSON, 1951). It has also been observed that physical factors, such as freezing the powdered tissue or excessive homogenization, cause a definite increase in the 'free' acetylcholine content of extracts. The study of possible variations in the 'free' fraction of acetylcholine *in vivo* would be hard in view of these technical difficulties.

The reduction of cholinesterase activity in the 'stimulated' *ep* mice is apparently a secondary change due to previous convulsions, but the reason remains to be further elucidated.

SUMMARY

1. A convulsive strain of mouse (*ep* strain) is described, in which convulsions can be induced in every adult animal by postural stimuli causing disequilibration.

2. The free ammonia level of the brain of *ep* mice is found to be 40 per cent lower than that of mice of control strains. A marked rise of brain ammonia can be induced in *ep* mice, both at the preconvulsive and convulsive stage, by applying postural stimulation.

3. Both the brain glutamine and glutamic acid of *ep* mice are 30 per cent lower than those of controls. In contrast, the γ-aminobutyric acid level of *ep* mice is 40–50 per cent higher. Postural stimulation, long enough in duration to cause convulsions in *ep* mice, does not cause significant changes in the brain glutamine, glutamic or γ-aminobutyric acids.

4. The level of total acetylcholine of *ep* mice is found to be 40–60 per cent higher than those of control strains. A significant fall of aceytlcholine is induced in response

VOL.
5
1959/6

to postural stimulation, both at the preconvulsive and convulsive stage. The fall is smaller in the previously 'non-stimulated' than in the previously 'stimulated' *ep* mice. The fall is not significant during the refractory period.

5. The rate of acetylcholine resynthesis in isolated brain tissues of *ep* mice is higher than that of the control strain. Cholinesterase activity of brain tissues is reduced in the previously 'stimulated' *ep* mice. There is no difference in the respiratory rate of brain slices from the different strains.

Acknowledgements—The authors are greatly indebted to Prof. Y. UCHIMURA and Prof. H. AKIMOTO for their valuable criticisms during the work. They would also like to thank Dr. K. IMAIZUMI for providing the *ep* mice and for many valuable suggestions, and Dr. H. HILLMAN for help in preparing the manuscript.

REFERENCES

BENITEZ D., PSCHEIDT G. R. and STONE W. E. (1954) *Amer. J. Physiol.* **176,** 488.
BERL S. and WAELSCH H. (1958) *J. Neurochem.* **3,** 161.
CONWAY E. J. (1947) *Microdiffusion Analysis and Volumetric Error* (2nd Ed.) Crosby, Lockwood, London.
CROSSLAND J., ELLIOTT K. A. C. and PAPPIUS H. M. (1955) *Amer. J. Physiol.* **183,** 32.
CROSSLAND J. and MERRICK A. J. (1954) *J. Physiol.* **125,** 56.
CROSSLAND J., PAPPIUS H. M. and ELLIOTT K. A. C. (1955) *Amer. J. Physiol.* **183,** 27.
DAWSON R. M. C. (1953) *Biochim. biophys. acta* **11,** 548.
DAWSON R. M. C. and RICHTER D. (1950) *Amer. J. Physiol.* **160,** 203.
ELLIOTT K. A. C. and HENDERSON N. (1951) *Amer. J. Physiol.* **165,** 365.
FELDBERG W. (1945) *J. Physiol.* **103,** 367.
IMAIZUMI K., ITO S., KUTUKAKE G., TAKIZAWA T., FUJIWARA K. and TUTIKAWA K. (1959) *Jikken-Dobutsu* (*Bulletin of the Experimental Animals*, Tokyo) **8,** 6.
KOLOUŠEK J., HORÁK F. and JIRÁČEK V. (1959) *J. Neurochem.* **4,** 175.
RICHTER D. and CROSSLAND J. (1949) *Amer. J. Physiol.* **159,** 247.
RICHTER D. and DAWSON R. M. C. (1948) *J. biol. Chem.* **176,** 1199.
ROBERTS E., HARMAN P. J. and FRANKEL S. (1951) *Proc. Soc. Exp. Biol., N.Y.* **78,** 799.
RUISSEAU J. P. DU, GREENSTEIN J. P., WINITZ M. and BIRNBAUM S. M. (1957) *Arch. Biochem. Biophys.* **68,** 161.
SCHWERIN P., BESSMAN S. P. and WAELSCH H. (1950) *J. biol. Chem.* **184,** 37.
SUTER C., KLINGMAN W. O., LACY O. W., BOGGS D., MARKS R. and COPLINGER C. B. (1958) *Neurology* **8,** Suppl. **1,** 117.
TOWER D. B. (1955) *Neurology* **5,** 113.
TSUKADA Y., TAKAGAKI G., SUGIMOTO S. and HIRANO S. (1958) *J. Neurochem.* **2,** 295.
VLADIMIROVA E. A. (1954) In *Biochimija Nervnoi Sistemi* (Edited by PALLADIN A. V.) p. 47, Akad. Nauk USSR, Kiev.
VRBA R. (1954) *Physiol. Bohemslov.* **3,** 394 (Quoted by VRBA R. (1955) *Nature, Lond.* **176,** 119 and 1258.)
VRBA R. and FOLBERGROVÁ J. (1959) In *Biochemistry of the Central Nervous System* (Edited by BRÜCKE F.) p. 131. Pergamon Press, London.

Journal of Neurochemistry, 1960, Vol. 5, pp. 370–378. Pergamon Press Ltd. Printed in Northern Ireland

THE INHIBITORY EFFECTS OF QUINONES AND DIHYDRIC PHENOLS ON GLUCOSE METABOLISM IN SUB-CELLULAR SYSTEMS OF BRAIN*

PAUL HOCHSTEIN and GERALD COHEN
Departments of Biochemistry and Psychiatry, College of Physicians and Surgeons, Columbia University, The Institute of Cancer Research, Columbia University, and The New York State Psychiatric Institute

(*Received* 30 *September* 1959)

VOL.
5
1959/6

IT IS now recognized that many disturbances of the nervous system result from alterations in specific biochemical reactions. Because glucose is essential to brain, factors which affect cerebral glucose utilization are often associated with profound changes in central nervous system function (TOWER, 1958). However, at the present time, little is known about the *in vivo* mechanisms which act to regulate glucose metabolism in brain. Hormones such as insulin, usually associated with peripheral carbohydrate metabolism, are thought to have no direct action on cerebral glucose metabolism (ELLIOTT, 1955). We have been concerned recently with the inhibitory action of various synthetic and naturally occurring quinones and dihydric phenols on the carbohydrate metabolism of brain. This communication deals with the effects of such compounds on the production of lactic acid in cell-free systems prepared from brain and other tissues. The experimental results demonstrate that in contrast to other tissues the glycolysis of brain is specially sensitive to inhibition. A possible role for quinones and diyhdric phenols in regulating the glucose utilization of brain *in vivo* is suggested.

METHODS

C57 mice were sacrificed by cervical dislocation, and the appropriate tissues rapidly removed and chilled. All subsequent operations prior to placing the reaction flasks at the experimental temperature of 30° were carried out in the cold. One volume of tissue was homogenized with 4 volumes of 0·25 M-sucrose in a glass tube fitted with a rotating plastic plunger. Homogenates were cleared of nuclei and cell debris by centrifugation for 1 min with a maximal force of 8,000 **g**. The cleared homogenates were then centrifuged for 30 min at 20,000 **g**. The supernatant fraction was thoroughly decanted, and the mitochondrial pellet washed once by resuspension and centrifugation. Finally, the volumes of the supernatant and mitochondrial fractions were both adjusted with sucrose solution to be equal to that of the original cleared homogenate. Of each fraction 0·5 ml was added to the reaction flasks. The total nitrogen content of the fractions was determined with Nessler's reagent (UMBREIT *et al.*, 1957).

The basic reaction medium used contained substrate, cofactors, and buffer in the following amounts: 20 μmoles glucose, 0·5 μmoles DPN, 4·0 μmoles ATP, 40 μmoles nicotinamide, 4·0 μmoles $MgSO_4 \cdot 7H_2O$, 11·5 μmoles K_2HPO_4, and 80 μmoles $KHCO_3$. The initial pH of the reaction medium was 7·6. The final fluid volume of the reaction flasks was 2·0 ml.

Manometric flasks were gassed with a mixture of 95% air–5% CO_2, or 95% N_2–5% CO_2. Inhibitors were added to the flasks before they were placed in the water bath, and the reactions were run for 30 min. Total gas exchange was followed manometrically during the experimental period, and linear reaction kinetics were observed in control flasks. Preliminary experiments showed that

* This work was aided by grants CY-2332 C4 and H-1045 C7 of the United States Public Health Service. The technical assistance of Mrs. A. DANILCHENKO is gratefully acknowledged.

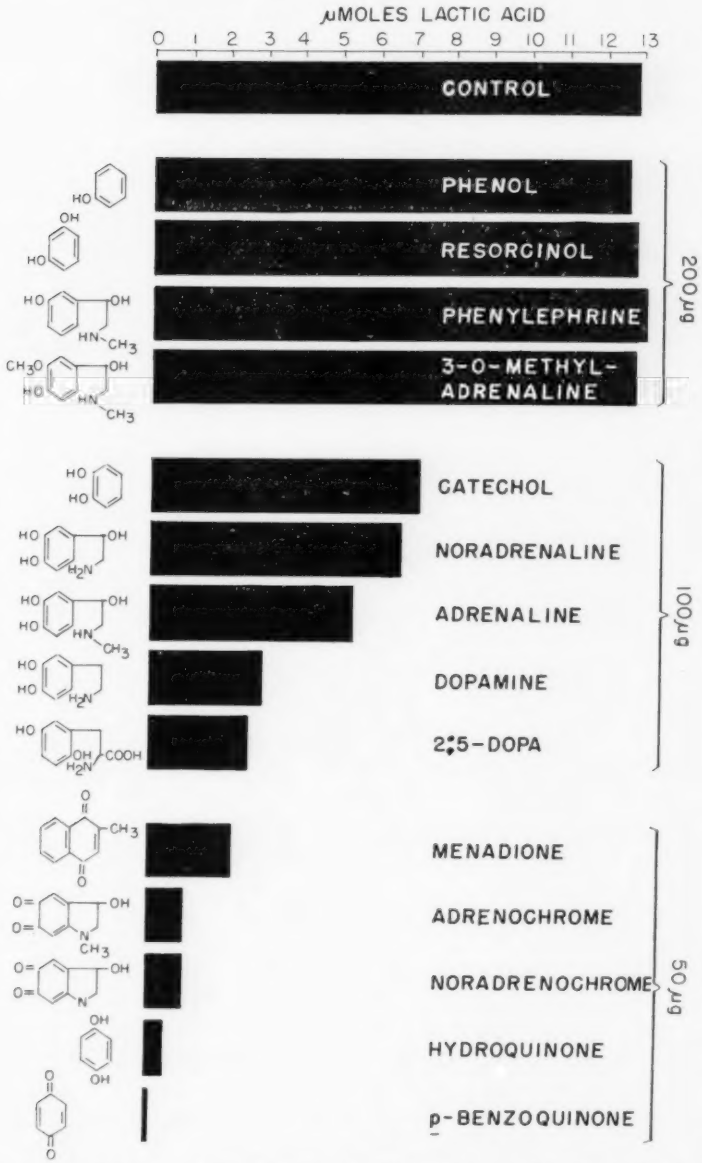


FIG. 1.—The effect of various phenols and quinones on aerobic glycolysis by brain homogenates in the standard system. Inhibitors were added to the reaction flasks in the amounts indicated. Total nitrogen content of the homogenate in each flask was 975 μg.

the total gas released could be accounted for by production of lactic acid (as determined chemically) in bicarbonate buffer. The experiments were terminated by the addition of 1·0 ml 10% TCA to the flasks, and their contents were analysed for lactic acid by the method of BARKER and SUMMERSON (1941).

L-Adrenaline, L-noradrenaline, 3:4-dihydroxyphenyl ethylamine (dopamine), 3-O-methyladrenaline (metanephrine), and *p*-hydroxyphenyl-N-methylethanolamine (phenylephrine) were obtained

from the Winthrop Laboratories. 2-Methyl-1:4-naphthoquinone (menadione) was obtained from Eastman Kodak Co., and 2:5-dihydroxyphenylalanine (2:5-dopa) from Merck and Co. These and other inhibitors were used without further purification. Adrenaline and noradrenaline were used as the bitartrate salts. Equivalent amounts of tartaric acid added to control flasks had no effect on glycolysis. Adrenochrome and noradrenochrome were prepared by the oxidation of adrenaline and noradrenaline with an excess of MnO_2 in 0·001 M-phosphate buffer, pH 6·8. The solutions were centrifuged, filtered, and used immediately. Blank solutions taken through the oxidation procedure had no inhibitory effects.

TABLE 1.—INHIBITION OF BRAIN GLYCOLYSIS UNDER AEROBIC AND ANAEROBIC CONDITIONS

In addition to the standard reaction mixture, each vessel also contained 20 μmoles HDP. Inhibitors were added to the main compartment of the Warburg flasks before the flasks were placed on the water bath and gassed. Figures in brackets are percentage inhibition. Total nitrogen content of the homogenate in each vessel was 840 μg.

	μmoles Lactic acid/30 min	
	Aerobic	Anaerobic
Control	12·6	13·8
Hydroquinone (2·5 μg)	8·6 (−32%)	12·8 (−7%)
Catechol (20 μg)	6·4 (−49%)	9·2 (−33%)
2:5-Dopa (20 μg)	10·8 (−14%)	12·6 (−9%)
Adrenaline (20 μg)	5·4 (−57%)	8·6 (−38%)
p-Quinone (1·25 μg)	10·0 (−21%)	12·4 (−10%)

RESULTS

All experiments have been repeated many times with essentially the same results. The results of single experiments performed in duplicate are presented for illustrative purposes. Duplicate determinations of lactic acid agreed within 5 per cent.

Inhibition of glycolysis in brain homogenates

Quinones and *o*- or *p*-dihydric phenols inhibited lactate formation from glucose, whereas mono-hydric and *m*-dihydric phenols did not (Fig. 1). For example, compounds such as *p*-quinone, catechol, and hydroquinone were active, whereas phenol and resorcinol were not. Of the compounds listed (Fig. 1) only those capable of undergoing reversible oxidation-reduction were inhibitory. It therefore seems likely that an oxidative mechanism was involved in the observed effects on glycolysis. In agreement with this conclusion is the fact that although inhibition took place under anaerobic as well as aerobic conditions, inhibition was always more pronounced in air rather than in an atmosphere of nitrogen (Table 1).

When cysteine was added to the reaction medium inhibition was greatly reduced. This point is illustrated in Table 2 where the percentage inhibition induced by various of the active compounds, in the presence and absence of cysteine, is shown. Although cysteine prevented inhibition, it did not to any great degree reverse inhibition produced prior to its addition. For example, the addition of cysteine to brain homogenates at zero time completely protected them against inhibition by adrenochrome (Expt. 2); on the other hand, the addition of cysteine after 30 min reduced the percentage inhibition only 9 per cent (Expt. 3).

VOL. 5 1959/6

In view of: (1) the protective effect of cysteine (Table 2); (2) the more pronounced inhibitory effects under aerobic conditions (Table 1); and (3) the greater activity of quinones as compared to almost all phenols (Fig. 1), it would appear that some oxidized form of these compounds, either the quinone itself or a free-radical intermediate is the active inhibitory species.

TABLE 2.—INHIBITION OF BRAIN GLYCOLYSIS IN THE PRESENCE AND IN THE ABSENCE OF CYSTEINE

In addition to the standard reaction mixture, HDP (20 μmoles) was added to the reaction flasks of Experiment 1. Cysteine (2·5 μmoles) was added to the flasks along with the inhibitors at zero time except in Experiment 3 where cysteine was added after 30 min. Total nitrogen contents of the homogenates in each vessel were: Experiment 1, 927 μg; Experiment 2, 850 μg; Experiment 3, 944 μg.

Expt.	μmoles Lactic acid/30 min	
	Without cysteine	With cysteine
1. *Aerobic atmosphere*		
Control	13·7	12·7
p-Quinone (25 μg)	1·8 (−87%)	12·1 (−5%)
Hydroquinone (25 μg)	3·6 (−74%)	11·7 (−8%)
2:5-Dopa (100 μg)	3·6 (−74%)	13·2 (0)
Adrenaline (100 μg)	5·4 (−61%)	10·3 (−19%)
Catechol (100 μg)	7·0 (−49%)	11·3 (−11%)
2. *Anaerobic atmosphere*		
Control	10·8	10·1
Adrenochrome (50 μg)	2·7 (−75%)	9·7 (0)
3. *Anaerobic atmosphere*		
Control	11·1	10·8
Adrenochrome (50 μg)	3·8 (−66%)	4·6 (−57%)

Site of inhibition of brain glycolysis. To localize the site of inhibitory action in the glycolytic scheme, various glycolytic intermediates were added to the reaction mixtures in addition to glucose. Table 3 illustrates the results of a typical experiment in which either glucose-6-phosphate (G-6-P), fructose-1:6-diphosphate (HDP) or 3-phosphoglyceric acid (3-PGA) was added to the medium in the presence and in the absence of the inhibitory compounds. In general, the percentage inhibition induced by each of the drugs was the same in the presence of G-6-P or HDP as it was with glucose alone. However, when 3-PGA was added to the reaction vessels, marked, although not usually complete, reversal of the inhibition of lactic acid production was observed. These data suggest that the primary site of inhibition is at one of the enzymes involved in the conversion of HDP to 3-PGA. To examine the possibility that inhibition results from interference with the function of DPN in HDP metabolism, excess amounts (8.0 μmoles) of DPN or DPNH were added to homogenates in the presence and in the absence of adrenaline or adrenochrome. No significant alleviation of inhibition was observed in these experiments; this result indicates that inhibition was not mediated through a direct effect on the oxidation state of DPN or through competition between inhibitor and coenzyme for some enzymic site.

It is of particular interest that, at the drug concentrations used, inhibition of lactate production was observed only in glycolytic systems in which mitochondria were present. Thus, as illustrated by the experiment with adrenochrome presented in Table 4 (lines 1 and 2), no inhibition was observed when the supernatant fraction of brain was incubated with adrenochrome in the absence of mitochondria; however, when mitochondria were added to the system profound inhibitory effects were observed. Similar results were obtained with the other inhibitory compounds.

TABLE 3.—INHIBITION OF BRAIN GLYCOLYSIS IN THE PRESENCE OF VARIOUS GLYCOLYTIC INTERMEDIATES

In addition to the standard reaction mixture, 20 μmoles of the glycolytic intermediates indicated in the Table were added to each flask. Figures in brackets are percentage inhibition. Total nitrogen contents of the homogenates in each flask were: Experiment 1, 814 μg; Experiment 2, 987 μg.

Expt.	μmoles Lactic acid/30 min			
	Glucose only	G-6-*P*	HDP	3-PGA
1. *Anaerobic atmosphere*				
Control	9·2	11·7	11·4	11·7
Adrenochrome (50 μg)	2·5 (−73%)	3·4 (−71%)	2·7 (−76%)	8·0 (−32%)
2. *Aerobic atmosphere*				
Control			13·6	14·2
Hydroquinone (25 μg)			6·5 (−52%)	12·5 (−12%)
Catechol (100 μg)			9·9 (−27%)	13·4 (−6%)
Adrenaline (100 μg)			6·9 (−49%)	11·8 (−17%)
2:5-Dopa (100 μg)			6·0 (−56%)	10·5 (−26%)

The special sensitivity of brain homogenates. Adrenaline, 2:5-dopa, and hydroquinone, at the concentrations indicated (Table 5), had marked effects on the glycolysis of brain, but generally small or no effects on the glycolysis of the other tissues tested. A similar differential sensitivity of brain homogenates to others of the compounds listed in Fig. 1 was observed. The striking difference between brain and other tissues appears to be of a quantitative nature since inhibition in homogenates of both liver and melanoma was observed when the concentration of inhibitor was increased 10- to 100-fold. For example, 200–400 μg of adrenochrome were required to inhibit melanoma homogenates to roughly the extent (50 per cent) that 5–10 μg adrenochrome inhibited in brain homogenates.

The reasons for the relative insensitivity of tissues other than brain appears to reside in factors present in the supernatant fractions of those tissues. For instance, when the mitochondria of liver or melanoma were incubated with the supernatant fraction of brain, inhibition of glycolysis was obtained at drug concentrations which inhibit whole brain homogenates. Conversely, when brain mitochondria were incubated with the supernatant fraction of liver or melanoma, inhibition was *not* observed at concentrations of drug which usually inhibit whole brain homogenates. This phenomenon is illustrated in Table 4. A concentration of adrenochrome which had no effect on the melanoma supernatant fraction (line 3) and only small effect on glycolysis by the recombined melanoma supernatant and mitochondrial fractions (line 4) markedly inhibited glycolysis of whole brain homogenates (line 2) or of

VOL. 5 1959/6

melanoma mitochondria combined with brain supernatant fraction (line 6). Conversely, the presence of melanoma supernatant fraction protected brain mitochondria from the main inhibitory effects of the drug (line 5).

It was observed during anaerobic experiments with the highly coloured (red) adrenochrome that bleaching of the colour occurred in tissues such as liver which were not subject to inhibition, whereas the colour persisted in brain where inhibition occurred. Thus, it appeared that the protection afforded other tissues was related to the

TABLE 4.—THE INHIBITORY EFFECT OF ADRENOCHROME ON ANAEROBIC GLYCOLYSIS BY VARIOUS FRACTIONS OF MOUSE BRAIN AND S91 MELANOMA

Figures in brackets are percentage inhibition. Total nitrogen contents of the fractions were: Brain supernatant, 410 μg, Brain mitochondria, 392 μg, Melanoma supernatant, 682 μg, melanoma mitochondria, 371 μg.

Source of mitochondria	Source of supernatant	μmoles Lactic acid/30 min	
		Control	Adrenochrome (100 μg)
———	Brain	3·2	3·1 (0)
Brain	Brain	13·4	1·5 (−89%)
———	S91 Melanoma	4·5	4·7 (0)
S91 Melanoma	S91 Melanoma	12·5	10·6 (−15%)
Brain	S91 Melanoma	14·6	12·9 (−12%)
S91 Melanoma	Brain	12·9	0·5 (−96%)

reducing capacity of the supernatant fraction, which appeared to be enzymically linked since bleaching occurred with greatest rapidity in the presence of glucose and all cofactors, whereas very much slower bleaching was noted in the presence of glucose but in the absence of the cofactors.

DISCUSSION

The site of inhibition. The data demonstrate a marked sensitivity of brain homogenate glycolysis to inhibition by quinones and *o*- and *p*-dihydric phenols. Many sulphydryl-dependent enzymes are inhibited by oxidizing agents such as quinones. For example, hexokinase (MEYERHOF and RANDALL, 1948) and succinic dehydrogenase (WOODFORD, 1959) of brain have been reported to be inhibited by adrenochrome. In our system, the primary site of inhibition appears to be located between fructose-1:6-diphosphate and 3-phosphoglyceric acid in the glycolytic scheme. The sensitivity of the enzyme glyceraldehyde-3-phosphate dehydrogenase to sulphydryl oxidizing agents (LIPMANN, 1942) makes it seem likely that the inhibition we observe is localized at this point.

The proposed site of inhibition in our system differs from that previously reported for adrenochrome by MEYERHOF and RANDALL (1948). They found that adrenochrome inhibited hexokinase of brain homogenates but not of yeast, and that inhibition was by-passed by using fructose-1:6-diphosphate as substrate or reversed by adding increasing amounts of ATP. In our experiments, all of the active compounds, including adrenochrome, inhibited glycolysis of brain to approximately the same extent with either glucose, glucose-6-phosphate, or fructose-1:6-diphosphate as substrate. Inhibition was not reversed by ATP. However, it should be noted that in systems in

which the production of ATP from glyceraldehyde-3-phosphate limits the rate at which glucose is phosphorylated, inhibition of glyceraldehyde dehydrogenase would result in an apparent inhibition of glucose phosphorylation which would be reversed by ATP (RACKER and KRIMSKY, 1948). Thus, the ultimate effect of glyceraldehyde-phosphate dehydrogenase inhibition may be diminished phosphorylation and an accumulation of glucose.

In the present experiments, inhibition was observed only in glycolysing systems to which mitochondria had been added. The addition of mitochondria to the soluble supernatant fraction of disrupted cells under the conditions of these experiments results in many-fold increases in glycolytic rate. Such increases in lactate production are in keeping with the suggestion made by DUBUY and his co-workers (HESSELBACH and DUBUY, 1953; DUBUY and HESSELBACH, 1956) that a major portion of the glycolytic enzymes is associated with the particulate rather than the soluble phase of broken cells. It is of special interest that, at the concentrations indicated, the compounds used in this study had no effect on glycolysis by the soluble fraction of cells. Marked effects, however, were observed when the mitochondria and supernatant fractions were recombined; this result suggests that inhibition takes place primarily at the mitochondrial level.

Inhibition of brain glycolysis in vivo. Current evidence indicates that glucose serves as a primary source of energy in brain through the formation of ATP. It seems reasonable therefore to assume that the rate of glucose utilization in various areas of brain may have important functional concomitants *in vivo*. Indeed, in extreme cases when the rate of glucose utilization is severely curtailed, cerebral dysfunction ensues. For example, when the availability of glucose to brain is markedly reduced as after the administration of large doses of insulin, coma develops. Similarly, gross neurological malfunction such as ataxia, opisthotonia, disturbance of equilibrium, coma, or convulsions can be induced through thiamine deficiency or by the administration of fluorocitrate or 2-desoxyglucose; each of these treatments blocks a specific step in the metabolism of glucose by brain (TOWER, 1958*a*, *b*). Although the pathway of rigorous logical argument leading from biochemical data at the subcellular level to behavioural effects in the intact animal contains serious gaps in knowledge, it has become generally accepted that the above functional defects are causally related to the corresponding metabolic blocks.

The administration of large doses of quinone or hydroquinone to animals has been reported to result in ataxia, convulsions, coma, and death (SEIFTER, 1946). In view of the potent inhibitory effects of quinones and dihydric phenols on brain glycolysis *in vitro*, as demonstrated in this present paper, we suggest that at least part of the functional defects observed may have been the direct result of inhibition of glucose metabolism in the central nervous system. Dihydric phenols and quinones may be derived as products of phenylalanine, tyrosine, or tryptophan metabolism. Thus glycolytic inhibitors of this type (not necessarily limited to those reported in this paper) may be produced *in vivo* in contrast to fluorocitrate and 2-desoxyglucose which are of exogenous origin. It is tempting to speculate that endogenous inhibitors gaining access to brain tissue may serve to modulate some aspect of cerebral function. Although the inhibition of sulphydryl-dependent enzymes by oxidizing agents such as quinones is a well known phenomenon, what is particularly striking in the experiments reported here is the singular susceptibility of brain tissue glycolysis (cf. Table 5).

VOL.
5
1959/6

Thus, because of the protective mechanisms present in other tissues (cf. Table 4), or the derivation of energy from substrates other than glucose, potential inhibitors in tissues other than brain need not produce functional effects. Furthermore, in the central nervous system where cells are highly differentiated with regard to function, inhibition of small groups of cells may result in marked qualitative aberrations; in tissues such as muscle, on the other hand, where all cells are working synchronously to perform the same function, a similar inhibition of small groups of cells may result in only a slight quantitative effect. We, therefore, draw attention to endogenous

TABLE 5.—THE INHIBITORY EFFECT OF ADRENALINE, 2:5-DOPA AND HYDROQUINONE ON GLYCOLYSIS BY VARIOUS MOUSE TISSUE HOMOGENATES

The experiments were run under aerobic conditions and the figures in brackets are percentage inhibition. HDP (20 μmoles) was added to each of the reaction flasks in addition to the standard reaction mixture.

Tissue	Total N/flask (μg)	μmoles Lactic acid/30 min			
		Control	Adrenaline (100 μg)	2:5-Dopa (100 μg)	Hydroquinone (50 μg)
Brain	1050	13·6	6·2 (−54%)	6·0 (−56%)	0·5 (−96%)
Liver	816	9·2	9·1 (0)	9·2 (0)	9·1 (0)
S91 Melanoma	980	11·9	12·0 (0)	11·9 (0)	12·1 (0)
Heart	790	9·4	—	9·4 (0)	7·5 (−20%)
Kidney	705	9·4	—	9·4 (0)	9·4 (0)

dihydric phenols and their corresponding quinones as possible mediators of some aspects of cerebral activity and of functional defects in the central nervous system.

SUMMARY

1. Various quinones (e.g. adrenochrome, menadione, *p*-quinone) and dihydric phenols (e.g. adrenaline, 2: 5-dopa, catechol) inhibited lactic acid formation from glucose in cell-free homogenates prepared from mouse brain.

2. Evidence is presented that inhibition took place through an oxidative mechanism primarily on the enzyme glyceraldehyde-3-phosphate dehydrogenase, at the mitochondrial level of cellular organization.

3. Brain homogenates were much more sensitive to inhibition than other tissues (e.g. liver, heart). The reason for the greater sensitivity of brain was found to reside in the absence of certain metabolic factors from the supernatant (free of mitochondria) fraction of brain.

4. A possible role for endogenous dihydric phenols and their corresponding quinones in regulating brain glucose metabolism and hence cerebral function is discussed.

REFERENCES

BARKER S. B. and SUMMERSON W. H. (1941) *J. biol. Chem.* **138,** 535.

DUBUY H. G. and HESSELBACH M. L. (1956) *J. Histochem. Cytochem.* **4,** 363.

ELLIOTT K. A. C. (1955) *Neurochemistry* (Edited by ELLIOTT K. A. C., PAGE I. H. and QUASTEL J. H.) Thomas, Springfield, Ill.

HESSELBACH M. L. and DUBUY H. G. (1953) *Proc. Soc. Exp. Biol., N.Y.* **83,** 62.

LIPMANN F. (1942) *A Symposium on Respiratory Enzymes* Univ. Wisconsin Press, Madison.

MEYERHOF O. and RANDALL L. O. (1948) *Arch. Biochem* **17,** 171.
RACKER E. and KRIMSKY I. (1948) *J. biol. Chem.* **173,** 519.
SEIFTER J. (1946) In *Biological Antioxidants* (Edited by MACKENZIE C. G.) Josiah Macy Jr. Foundation, New York.
TOWER D. B. (1958*a*) In *Biological and Biochemical Basis of Behavior* (Edited by HARLOW H. F. and WOOLSEY C. N.) Univ. Wisconsin Press, Madison.
TOWER D. B. (1958*b*) *J. Neurochem.* **3,** 185.
UMBREIT W. W., BURRIS R. H. and STAUFFER J. F. (1957) *Manometric Techniques* Burgess Publishing Co., Minneapolis.
WOODFORD V. R. (1959) *Canad. J. Biochem. Physiol.* **37,** 873.

VOL.
5
1959/6

Journal of Neurochemistry 1960, Vol. 5, pp. 379 to 382. Pergamon Press Ltd. Printed in Northern Ireland

A COMPARISON BETWEEN THE INCORPORATION OF ^{14}C FROM GLUCOSE INTO N-ACETYL-L-ASPARTIC ACID AND ASPARTIC ACID IN BRAIN PERFUSION EXPERIMENTS*

R. U. MARGOLIS, S. S. BARKULIS† and A. GEIGER
Department of Psychiatry, University of Illinois College of Medicine
Chicago, Illinois

(*Received* 28 *December* 1959)

OL.
5
9/60

FREE N-acetyl-L-aspartic acid has recently been shown to occur in mammalian and avian brain tissue at a concentration of 80–110 mg/100 g, and at one-fifth of this level in the brain of the turtle. It could not be detected in human cerebrospinal fluid, cat gastrocnemius or urine, and not more than 1 and 3 mg/100 g were found in the liver and kidney, respectively (TALLAN *et al.*, 1956, 1957). It was not detectable in the brain of the frog, horseshoe crab, or lobster.

The present study was concerned with the rate of incorporation by the perfused cat brain of ^{14}C from glucose into acetylaspartic acid as compared to that into aspartic acid, and was part of a larger study (BARKULIS *et al.*, 1960) devoted to determining the rate of incorporation of [^{14}C] glucose into a number of amino acids, among them glutamic acid and aspartic acid. The *in situ* brain perfusion method of GEIGER and MAGNES (1947) was used, with the addition of uniformly ^{14}C-labelled glucose to the perfusion blood.

JACOBSON (1958) has previously reported a small conversion of [^{14}C] glucose and [^{14}C] aspartic acid to acetylaspartic acid in mouse brain homogenates and in the brain of the living mouse.

EXPERIMENTAL

Brain cortex samples were taken periodically up to 75 min after the addition of [^{14}C] glucose to the perfusion blood, and perchloric acid extracts were prepared in the manner previously described (BARKULIS *et al.*, 1960). The extracts were neutralized with 6 N- and 2 N-KOH, the precipitated potassium perchlorate was sedimented and washed, and the supernatant fluid evaporated to dryness in a rotatory evaporator under vacuum. The dried extracts were dissolved in 2·5 ml of 0·1 N-HCl and placed on a 0·9 × 7·5 cm column of Dowex 50-X-4 (200–400 mesh). 30 ml of water were added and the effluent, containing chiefly glucose, acetylaspartic acid, lactic acid, and nucleotides was collected. The acidic and basic amino acids were eluted with 30 ml of 6 N-HCl for further studies.

In the water effluent from the Dowex 50-X-4 column, acetylaspartic acid was determined by two methods. In one, the acetylaspartic acid was hydrolysed (90%) (after reduction to a convenient volume in a rotatory evaporator at room temperature) by adding enough HCl to make the solution 2 N and heating for 30 min in a boiling water bath (TALLAN *et al.*, 1956). This was taken to dryness on the rotatory evaporator at room temperature and re-dissolved in 2·5 ml of 0·5 N-acetic acid, and the aspartic acid separated by chromatography on a 0·9 × 30 cm column of Dowex 1-X-8 (200–400 mesh).

In the second method, the water effluent was evaporated at room temperature without previous hydrolysis, and the syrupy residue was re-dissolved in 2·5 ml of 0·2 M-sodium acetate buffer at pH

* This work was supported by a grant from the National Institute of Neurological Diseases and Blindness, B-413(C6) and by the National Multiple Sclerosis Society.

† Present address: Microbiology Division, Ciba Pharmaceutical Products, Inc. Summit, New Jersey.

5·0. Acetylaspartic acid as such was separated from the mixture by MOORE and STEIN's (1954) gradient elution technique. Elution proceeded with a sodium acetate buffer of increasing ionic strength by allowing 1 l. of 2 N-sodium acetate to flow into a mixing chamber containing 140 ml (instead of the 150 ml of MOORE and STEIN's method) of 0·2 N-sodium acetate at pH 5·0.

In both procedures the effluent was collected in 2·6 ml fractions at a flow rate of 5–7 ml per hr. Aspartic acid emerges at 75–90 ml in an eluant of 0·5 N-acetate. Ninhydrin analyses were performed with the method of ROSEN (1957); when acetylaspartic acid was separated as such, alternate fractions were hydrolysed (90%) for 30 min in a boiling water bath with 1·3 ml of 6 N-HCl, and the hydrolysates neutralized with 2 N-NaOH prior to ninhydrin analysis.

In the method used for separation of acetylaspartic acid as such, the glucose is completely eluted by the first 50 ml of sodium acetate solution.

Acetylaspartic acid was synthesized with the procedure of BARKER (1953); a viscous syrup which would not crystallize resulted. The crystalline *p*-dinitrobenzyl ester was prepared by treatment with α-bromo-*p*-nitrotoluene according to the general method outlined by SHRINER, FUSON and CURTIN (1956) for the esterification of acids. It had a melting point of 123–124° which compares well with that given in the literature. With sodium acetate buffer as eluant, the synthetic acetylaspartic acid when applied to the column, emerged at 85–100 ml of effluent, as did the unknown (effluent ninhydrin positive after hydrolysis).

Portions of 1 ml of the pooled fractions were transferred into the 5 dram glass vials (obtained from Packard Instrument Co., La Grange, Illinois) used for liquid scintillation counting and dried in a vacuum desiccator at room temperature. The samples were dissolved in 1 ml of a methanolic solution of the hydroxide form of the quaternary amine, Hyamine,* prepared according to the method described by PASSMAN *et al.* (1956). A 'solvent system', consisting of 4 g 2:5-diphenyloxazole (PPO) and 100 mg 1:4-di(2-(5-phenyloxazolyl))benzene (POPOP) diluted to 1 l. with toluene, was prepared, of which 10 ml were used for each sample. The samples were cooled to about 5° and counted in a Packard Tri-Carb Model 314 Liquid Scintillation Spectrometer.

RESULTS

In the course of one hour's perfusion with uniformly ^{14}C-labelled glucose, aspartic acid becomes highly labelled (2,000–5,000 counts/min per μmole) (BARKULIS *et al.*, 1960). The incorporation of the ^{14}C into acetylaspartic acid proceeds at a much slower rate (30–80 counts/min per μmole) during the same period. Acetylaspartic acid, in brain cortex samples taken 60 to 73 min after the addition of the [^{14}C] glucose to the perfusion fluid, showed only 1·17 to 2·12 per cent of the activity of the aspartic acid measured in the same brain samples (Table 1). These results are in complete agreement with those obtained in experiments (Table 2) in which the activity of the acetylaspartic acid was determined by analysis of the aspartic acid liberated upon hydrolysis.

In order to compare these results with those obtained under more normal conditions, in one experiment [^{14}C] glucose was infused at a constant rate into an intact cat in 'Nembutal' narcosis, the activity of glucose in the blood being maintained fairly constant for 80 min. In this experiment, the activity of the acetylaspartic acid reached approximately 5 per cent (81 counts/min per μmole) of that of the aspartic acid from the same brain sample. This incorporation rate is of the same order of magnitude as that obtained during perfusion.

In perfusion experiments neither 'Nembutal' narcosis nor 'Metrazol'-induced convulsions produced any significant difference in either the total quantities of the free amino acids present or in the pattern of appearance of radioactivity in any of the amino acids determined (BARKULIS *et al.*, 1960). This is now found to be true for acetylaspartic acid as well.

* *Para*-di*iso*butyl crezoxy methoxy ethyl dimethyl benzyl ammonium chloride monohydrate = Hyamine —10x, Rohm and Haas Co., Philadelphia, Pa.

VOL. 5 1959/6

TABLE 1.—Concentration and specific activity of aspartic acid and acetylaspartic acid determined as such

Sample of brain cortex	Weight of sample (g)	Duration of perfusion with ^{14}C-labelled glucose (min)	Drugs used during experiment	μmoles Aspartic acid per g brain	Counts/min per μmole aspartic acid	μmoles Acetyl aspartic acid per g brain	Counts/min per μmole acetyl aspartic acid	Activity of acetyl-aspartic acid expressed as % of that of aspartic acid
5–19–59	1·162	72	Nembutal	1·51	2170	4·65	46	2·12
6–10–59	0·723	60	None	2·04	2730	6·07	32	1·17
6–4–59	1·028	70	Nembutal	1·59	4480	6·12	59	1·35
6–24–59	1·892	73	Metrazol	1·51	3174	3·79	65	2·05

TABLE 2.—Concentration and specific activity of aspartic acid and acetylaspartic acid determined as aspartic acid after hydrolysis

3–3–59	1·071	52	Metrazol	2·21	6970	5·88	57	1·22
4–7–59	1·788	62	Metrazol	1·71	5200	4·79	29	1·79
6–10–59	1	60	None	2·04	2730	6·10	56	2·05
3–3–59	1	57	Metrazol	2·21	6970	5·43	76	1·09
6–24–59	1	75	Metrazol	1·51	3174	4·27	42	1·32

Note: Samples 6–10–59, 3–3–59, and 6–24–59 were 1 g portions taken from the homogenates of a whole hemisphere.

DISCUSSION

TALLAN (1957) has reported that the concentration of acetylaspartic acid in bovine nervous tissue ranges from 124 mg/100 g in the cerebral grey matter to 48 mg/100 g in the spinal roots, and that its concentration is twice as high in the cerebral grey matter as in the white matter. Our figures for the concentration of acetylaspartic acid in brain cortex are approximately the same as those of TALLAN *et al.* (1956).

The mode of synthesis of acetylaspartic acid has recently been investigated by GOLDSTEIN (1959*a*, *b*), who found that rat brain slices incubated in a glucose-phosphate medium containing 0·05–0·10 M-KCl incorporated ^{14}C from acetate, pyruvate, or aspartate into acetylaspartic acid. He partially purified from this tissue an enzyme, L-aspartic acetylase, which catalysed the acetylation of L-aspartic acid by acetyl-coenzyme A to form N-acetyl-L-aspartic acid.

The following reactions were proposed by GOLDSTEIN (1959*b*) for the bio-synthesis of acetylaspartic acid in the brain:

(1) Acetate + ATP $\rightleftarrows$ acetyl-AMP + PP

(2) Acetyl-AMP + CoA-SH $\rightleftarrows$ acetyl-CoA + AMP

(3) Acetyl-CoA + L-aspartate $\rightarrow$ acetylaspartate + CoA-SH

Because of the extremely low rate of incorporation of ^{14}C from glucose into the aspartyl moiety of acetylaspartic acid, as contrasted with the relatively high incorporation rate into free aspartic acid in our experiments, it must be assumed that if the proposed mechanism of bio-synthesis is correct, reaction 3 proceeds very slowly in the living brain. If this is correct, most of the acetylaspartic acid must have been present in the brain before the experiments and could not have been labelled through pathways of amino acid metabolism, such as transamination.

Acetylaspartic acid may contribute (at a concentration of 100 mg/100 g) about 11 μequiv./g to the 90 μequiv./g so-called anion deficit known to exist in nervous tissue (MCILWAIN, 1959).

KOREY *et al.* (1951) have found that choline acetylase from the head ganglion of the squid is capable of utilizing the α-N-acetyl derivatives of glycine, L-alanine, and L-lysine as substitutes for acetate in the production of acetylcholine. The question of whether or not mammalian choline acetylase can carry out similar reactions and of the part that these might play in the normal formation of acetylcholine is still unanswered.

VOL.
5
1959/6

SUMMARY

It was found that the rate of incorporation of ^{14}C from uniformly labelled glucose into N-acetyl-L-aspartic acid during brain perfusion experiments averaged only 1–5 per cent of its incorporation rate into aspartic acid in the brain.

REFERENCES

BARKER C. C. (1953) *J. chem. Soc.* 453.
BARKULIS S. S., GEIGER A., KAWAKITA Y. and AGUILAR V. (1960) *J. Neurochem.* **5,** 339.
BAXTER C. cited in JACOBSON (1958).
GEIGER A. and MAGNES J. (1947) *Amer. J. Physiol.* **149,** 517.
GOLDSTEIN F. B. (1959*a*) *Biochim. biophys. acta* **33,** 583.
GOLDSTEIN F. B. (1959*b*) *J. biol. Chem.* **234,** 2702.
JACOBSON K. B. (1958) *Fed. Proc.* **17,** 248.
KOREY S. R., DEBRAGANZA B. and NACHMANSOHN D. (1951) *J. biol. Chem.* **189,** 705.
MCILWAIN H. (1959) *Biochemistry and the Central Nervous System* (2nd Ed.) Churchill, London.
MOORE S. and STEIN W. H. (1954) *J. biol. Chem.* **211,** 893.
PASSMAN J. M., RADIN N. S. and COOPER J. A. D. (1956) *Analyt. Chem.* **28,** 484.
ROSEN H. (1957) *Arch. Biochem. Biophys.* **67,** 10.
SHRINER R. L., FUSON R. C. and CURTIN D. Y. (1956) *The Systematic Identification of Organic Compounds* (4th Ed.) Wiley, New York.
TALLAN H. H. (1957) *J. biol. Chem.* **224,** 41.
TALLAN H. H., MOORE S. and STEIN W. H. (1956) *J. biol. Chem.* **219,** 257.

Journal of Neurochemistry, 1960, Vol. 5, pp. 383 to 384. Pergamon Press Ltd. Printed in Northern Ireland

PRELIMINARY NOTE

The interaction of strandin with basic proteins*

(*Received* 7 *March* 1960)

In a previous communication (Harris and Saifer, 1960), we noted that the metachromasy of strandin is suppressed by protamine sulphate. Therefore, the possibility of interaction between strandin and the basic proteins, lysozyme and protamine, was investigated.

Strandin was prepared from cortical gray matter of normal human brain by the method of Folch, Lees and Sloane-Stanley (1957). The preparation was analysed by the neuraminic acid method of Saifer and Gerstenfeld (1957), a mean value of 30·5 mg of neuraminic acid per 100 mg of strandin being assumed (Folch and Lees, 1959). Interaction was measured by a turbidimetric procedure.

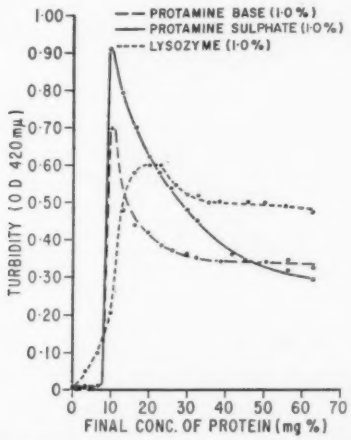


Fig. 1.—The effects of adding varying portions of 1·0% solutions of protamine sulphate or protamine base to 3 ml volumes of strandin (0·32 mg/ml), and of adding portions of 1·0% lysozyme solution to a 3 ml volume of strandin containing 0·11 mg/ml. Turbidimetric measurements (read as optical densities) were made at 26° and at 420 mμ in a Bausch and Lomb Spectronic 20, Spectrophotometer. The final volume was 3·2 ml, and the final pH, at highest protein concentration, was 7·4 for protamine base, 3·9 for protamine sulphate, and 6·5 for lysozyme.

When varying amounts of protamine base or protamine sulphate were added to solutions of strandin (Fig. 1), there was no interaction with small amounts, a large, maximal interaction with slightly larger amounts, and a decreasing degree of interaction as the amounts of protein were further increased. With lysozyme, there was some interaction with the smallest amount studied, and a more gradual increase in the degree of interaction to a maximum and smaller decrease from the maximum, as the amounts were increased, than with the other proteins.

The interaction is sensitive to temperature. Thus, the turbidity of a protamine sulphate–strandin system (3 ml of aqueous solution containing 0·1 ml of 1·0% protamine sulphate and 0·3 ml of 0·32% strandin) increased with decreasing temperature over the range of 55–6°. With a lysozyme system

* Supported by a grant from the National Tay-Sachs' Association.

(3 ml aqueous solution containing 0·1 ml of 1·0% lysozyme, and 0·1 ml of 0·32% strandin), the turbidity increased with increasing temperature.

The interaction of these systems was not significantly affected by pH changes in the range pH 5 to pH 10. The amount of interaction decreased beyond this range in either direction.

All metal salts reduced the turbidity of a protamine–strandin system (Fig. 2), and the phenomenon was valence-dependent, the order of effectiveness being: tervalent > bivalent > univalent. No significant pH changes occurred. Similar results were obtained with the lysozyme–strandin system.

The turbidity of both systems was reduced by addition of quaternary ammonium salts. Cetyltrimethylammonium bromide (0·01 M) caused complete loss of turbidity. Both trimethylammonium

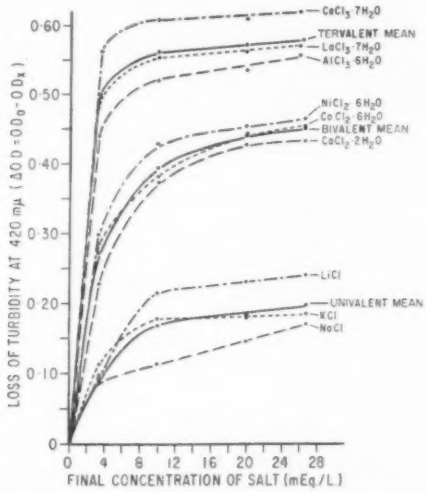


FIG. 2.—Loss of turbidity (ΔOD) due to interaction of metal salts with a protamine–strandin system (3 ml of aqueous solution containing 0·1 ml of 1·0% protamine sulphate and 0·3 ml of 0·32% strandin). The final volume was 3 ml. ΔOD is the difference between the initial optical density (OD_0) and the optical density at any given concentration of salt (OD_x), i.e. $\Delta OD = (OD_0 - OD_x)$.

bromide (0·1 M) and acetylcholine bromide (0·1 M) were far less effective. No significant pH changes occurred.

Addition of chondroitin sulphate (25 mg per 100 ml) increased the turbidity of the protamine–strandin system and decreased that of the lysozyme–strandin system.

The turbidity of both systems was reduced by the addition of any one of three aliphatic alcohols; *n*-propanol was more active than either methanol or ethanol.

The results presented indicate that the interaction between strandin and basic proteins is dependent on the physico-chemical environment. The nature of this interaction, i.e. whether involving complex or salt formation, will be the subject of a future communication.

ALFRED F. HARRIS
ABRAHAM SAIFER

Biochemistry Department,
Isaac Albert Research Institute
of the Jewish Chronic Disease Hospital,
Brooklyn 3, *N.Y.*

REFERENCES

FOLCH J. and LEES M. (1959) *Amer. J. Dis. Child.* **97,** 730.
FOLCH J., LEES M. and SLOANE-STANLEY G. H. (1957) *J. biol. Chem.* **226,** 497.
HARRIS A. F. and SAIFER A. (1960) *J. Neurochem.* **5,** 218.
SAIFER A. and GERSTENFELD S. (1957) *J. Lab. clin. Med.* **50,** 17.

VOL.
5
1959/6

Journal of Neurochemistry, 1960, Vol. 5, p. 385. Pergamon Press Ltd. Printed in Northern Ireland

ANNOUNCEMENT

Commission for Neuropathology

AT A meeting in Paris on 24th October 1959, a Commission for Neuropathology was established by the World Federation of Neurology. The members of the Commission are:—

J. BERTRAND (France)
E. CHRISTENSEN (Denmark)
P. B. DIEZEL (Germany)
W. GIRARD (France)
W. HAYMAKER (U.S.A)
A. C. LOKEN (Norway)
F. LÜTHY (Switzerland)
W. H. MCMENEMEY (U.K.)
E. OSETOWSKA (Poland)
G. PETERS (Germany)
F. SEITELBERGER (Austria)
P. SOURANDER (Sweden)
J. O. TRELLES (Peru)
W. J. C. VERHAART (Holland)

The Commission will serve as a consultative body within the World Federation of Neurology. It will also promote the exchange of scientific information between neuropathologists in different countries and consider the protection of their basic interests as members of an independent medical discipline. It emphasizes the need for independent full-time posts in neuropathology.

The Commission is interested in securing the maintenance of valuable collections of neuropathological specimens. It will sponsor the publication of a new international journal of neuropathology. Those wishing to refer suggestions to the Commission should contact the Secretary, Prof. F. SEITELBERGER at the permanent secretariat in the Institute of Neurology of the University of Vienna (Obersteiner Institut), Vienna 9, Schwarzspanierstrasse 17.

Journal of Neurochemistry, 1960, Vol. 5, p. 386. Pergamon Press Ltd. Printed in Northern Ireland

Papers to be published in future issues

H. HYDÉN and N. PIGON: A cytophysiological study of the functional relationship between oligodendroglial cells and nerve cells of Deiters' nucleus

I. J. YOUNG and L. G. ABOOD: Histological demonstration of hyaluronic acid in the central nervous system

D. A. RAPPOPORT, R. R. FRITZ, J. R. ALLEN and A. MORACZEWSKI: Nucleoside metabolism in brain extracts from rat, rabbit and guinea pig

W. LEVINE, A. GIUDITTA, S. ENGLARD and H. J. STRECKER: Brain diaphorases

LASCA H. BOGDANOVE and GEORGE CLARK: The encephalitic activity of some extractives of tissue from the central and peripheral nervous systems

WALTER L. NELSON and CYRUS P. BARNUM: The effect of diisopropylphosphorofluoridate (DFP) on mouse brain phosphorus metabolism

H. G. KNAUFF and F. BOCK: Über die freien Gehirnaminosäuren und das Äthanolamin der normalen Ratte, sowie über das Verhalten dieser Stoffe nach experimenteller Insulinhypoglykämie

A. LOWENTHAL, M. VAN SANDE and D. KARCHER: The differential diagnosis of neurological diseases by fractionating electrophoretically TH CFS γ-globulins

D. M. ROBERTSON: The electrophoretic distribution of the lipoproteins of the supernatant fraction of rat whole brain

E. GROSSI, P. PAOLETTI and R. PAOLETTI: The *in vitro* and *in vivo* effects of chlorpromazine on brain lipid synthesis

E. OKUHAR and M. YASUDA: Studies on conjugated lipids—VIII. Isolation of a dihydrocerebroside from human brain.

N. WEINER: The distribution of monoamine oxidase and succinic oxidase in brain

D. R. CURTIS and J. C. WATKINS: The excitation and depression of spinal neurones by structurally related amino acids.

F. CORRIDORI, T. CREMONA and G. TAGLIABUE: Glutamic-oxalacetic transaminase and lactic dehydrogenase activities in brain tumour homogenates.

KÅGE KJELLIN: The determination of copper in small quantities of cerebrospinal fluid by neutron activation analysis.

V. T. NACHMIAS: Amine oxidase and 5-hydroxytryptamine in developing rat brain.

VOL.
5
1959/6

OL.
5
9/60